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# Solid-phase extraction based sample preparation for the determination of drug and organic pollutant residues

*A thesis submitted in fulfillment of the requirement for the degree of*

*Master of Science in Chemistry*

**By**

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# Dedication

To my mom, with all my love…

# Acknowledgements

Prof. Nelson Torto: Thank you Prof for believing that 'I can', I will forever be grateful. You have steered my academic decisions and I am the person that I am now because of your unequalled mentoring and encouragement. I am extremely honored to be one of your students.

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To my family and friends: Thank you for giving me the strength to soldier on…

# Abstract

This thesis presents solid phase extraction (SPE) methodologies based on mixed-mode polymeric sorbents; a mixed mode strong anion exchanger (Agilent SampliQ SAX) and a mixed mode strong cation exchanger (Agilent SampliQ SCX). Furthermore, dispersive-SPE based on a quick, easy, cheap, effective, rugged and safe (QuEChERS) method was assessed for applicability in the determination of drug residues. The mixed-mode polymeric sorbents were evaluated for the simultaneous fractionation of drugs that exhibit diverse polarities with acidic, basic and neutral functionalities in biological matrices (plasma and urine). The polymeric skeleton of these sorbents entails an exchanger group and therefore provides two retention mechanisms, strong cation or anion exchange retention mechanisms with hydrophobic interactions. It was demonstrated that with a sequential elution protocol for sample clean-up analytes were fractionated into acidic, basic and neutral classes. The SAX was employed for analysis of ketoprofen, naproxen (acidic drugs), nortriptyline (basic) and secobarbital (neutral) from urine sample. The SCX was used for fractionating phenobarbital, p-toluamide (acidic), amphetamine, m-toluidine (basic) and acetaminophen (neutral drug) from plasma sample. QuEChERS method was employed for quantitative determination of 16 polycyclic aromatic hydrocarbons (PAHs) from fish fillets and soil; 9 sulfonamides (SAs) from chicken muscles and acrylamide (AA) in cooking oil. The analyte recoveries ranged from 79.6 - 109% with RSDs ranging from 0.06 - 1.9% at three different fortification levels. Good linearity ($r^2$ > 0.9990) was attained for most analytes. The limits of detection and quantification ranged from 0.03 - 0.84 µg/ml and 0.81 - 1.89 µg/ml respectively for analytes in biological samples. LODs and LOQs for analytes in food and environmental samples ranged from 0.02 to 0.39 and 0.25 to 1.30 ng/g respectively.

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# List of Abbreviations

| | | |
|---|---|---|
| 1. | %*R* | Percentage recovery |
| 2. | µg | Microgram |
| 3. | µl | Microliter |
| 4. | AA | Acrylamide |
| 5. | Ace | Acenaphthene |
| 6. | Acy | Acenaphthylene |
| 7. | ADS | Alkyl-diol-silica |
| 8. | AEDs | Antiepileptic drugs |
| 9. | Ant | Anthracene |
| 10. | AOAC | Association of Analytical Communities |
| 11. | BaA | 1,2-benza[a]anthracene |
| 12. | BeA | Benzo[e]acenaphthylene |
| 13. | BeP | Benzo[e]pyrene |
| 14. | Bghi | Benzo[g,h,i]perylene |
| 15. | BkF | Benzo[k]fluoranthene |
| 16. | C | Concentration |
| 17. | $C_{18}$ | Octadecyl bonded silica |
| 18. | CBD | Cannabidiol |
| 19. | CE | Capillary electrophoresis |
| 20. | $CH_3CN$ | Acetonitrile |
| 21. | $CH_3COONa$ | Sodium acetate |
| 22. | $CH_3OH$ | Methanol |
| 23. | Chr | Chrysene |
| 24. | CID | Collision-induce dissociation |

| | | |
|---|---|---|
| 25. | CN | Cyanopropyl bonded silica |
| 26. | $C_o$ | Initial concentration of the analyte in sample |
| 27. | d- SPE | Dispersive solid phase extraction |
| 28. | DahA | Dibenzo[a,h]anthracene |
| 29. | DEA | Drug Enforcement Administration |
| 30. | DNA | Deoxyribonucleic acid |
| 31. | EC | End capped |
| 32. | Em | Emission |
| 33. | Enq. | Equation |
| 34. | ESI | Electrospray ionisation mode |
| 35. | EU | European Union |
| 36. | Ex | Excitation |
| 37. | FDA | Food and Drug Administration |
| 38. | Fig. | Figure |
| 39. | FLD | Fluorescence detection |
| 40. | Fln | Fluoranthene |
| 41. | Flu | Fluorene |
| 42. | GC - MS | Gas chromatography–mass spectrometry |
| 43. | GC | Gas chromatography |
| 44. | GFF | Glycine-L -phenylalanine-L -phenylalanin |
| 45. | HAc | Glacial acetic acid |
| 46. | HLB | Hydrophilic-lipophilic balance |
| 47. | HPLC-DAD | High performance liquid chromatography-diode array detector |
| 48. | IAE | Immunoaffinity extraction |
| 49. | InP | Indeno[1,2,3-cd]pyrene |

| | | |
|---|---|---|
| 50. | IS | Internal standard |
| 51. | ISRP | Internal surface reversed-phase |
| 52. | K | Nernst Distribution |
| 53. | *k* | retention factor of the analyte |
| 54. | $K_2HPO_4$ | Potassium hydrogen phosphate |
| 55. | $K_{fs}$ | Partition coefficient for the analyte between fibre coating |
| 56. | $KH_2PO_4$ | Potassium dihydrogen phosphate |
| 57. | KOH | Potassium hydroxide |
| 58. | $K_{ow}$ | Octanol-water distribution coefficient |
| 59. | $K_{PDMS/w}$ | Distribution coefficient between PMDS and water |
| 60. | LC | Liquid chromatography |
| 61. | LD | Liquid desorption |
| 62. | LLE | Liquid–liquid extraction |
| 63. | LLLME | Liquid–liquid–liquid micro-extraction |
| 64. | LOD | Limit of detection |
| 65. | LOQ | Limit of quantification |
| 66. | LPME | Liquid-phase micro-extraction |
| 67. | LVI | large-volume injection |
| 68. | m/z | mass-to-charge ratio |
| 69. | MASE | Membrane-assisted solvent extraction |
| 70. | MeCN | Acetonitrile |
| 71. | MeOH | Methanol |
| 72. | $MgSO_4$ | Magnesium Sulphate |

| | |
|---|---|
| 73. MIPs | Molecularly imprinted polymers |
| 74. mM | Milli-molar |
| 75. $m_{PDMS}$ | Mass of the solute in the PDMS phase |
| 76. MRL | Maximum residue limits |
| 77. MRM | Multiple reaction monitoring |
| 78. MS/MS | mass spectrometry/ mass spectrometry |
| 79. $m_w$ | Mass of the solute in the aqueous phase |
| 80. n | Number of cycle |
| 81. N | Mass of the analyte adsorbed by the fibre coating |
| 82. *N* | Number of plates |
| 83. NaCl | Sodium chloride |
| 84. NaOAc | Sodium acetate |
| 85. Nap | Naphthalene |
| 86. NBD | Nitrobenz-2,1,3-oxadiazole |
| 87. ng | Nanograms |
| 88. $NH_2$ | Aminopropyl bonded silica |
| 89. nm | Nanometer |
| 90. NSAIDs | Non-steroidal anti-inflammatory drugs |
| 91. PA | Polar polyacrilate |
| 92. PAHs | Polycyclic aromatic hydrocarbons |
| 93. PDMS | Polydimethylsiloxane |
| 94. PH | Phenyl bonded silica |
| 95. Phe | Phenanthrene |
| 96. PLE | Pressurized liquid extraction |

| | | |
|---|---|---|
| 97. | PLEHW | Pressurized liquid extraction with subcritical heated water |
| 98. | PSA | Primary-secondary amine |
| 99. | PVDF | Polyvinylidene fluoride |
| 100. | Pyr | Pyrene |
| 101. | QuEChERS | Quick, Easy, Cheap, Effective, Rugged, and Safe |
| 102. | $r^2$ | Correlation coefficient |
| 103. | RAM | Restricted access material |
| 104. | REM | Rapid eye movement |
| 105. | RNA | Ribonucleic acid |
| 106. | rpm | Revolution per minute |
| 107. | RSD | Relative standard deviation |
| 108. | S/N | Signal-to-noise |
| 109. | SAs | Sulfonamides |
| 110. | SAX | Strong anion exchange |
| 111. | SBSE | Stir-bar sorptive extraction |
| 112. | SCX | Strong cation exchange |
| 113. | SDME | Single-drop micro-extraction |
| 114. | SEC | Size-exclusion chromatography |
| 115. | SFE | Supercritical fluid extraction |
| 116. | Si | Silica gel |
| 117. | SIM | Selected ion monitoring |
| 118. | SLME | Supported liquid membrane extraction |
| 119. | SPE | Solid phase extraction |
| 120. | SPME | Solid phase micro-extraction |

| | | |
|---|---|---|
| 121. | SPMEM | Solid phase micro-extraction membrane |
| 122. | SSHs | Steroid sex hormones |
| 123. | TD | Thermal desorption |
| 124. | THC | Tetrahydrocannabino |
| 125. | USA | United States of America |
| 126. | USEPA | United States Environmental Protection Agency |
| 127. | UV | Ultraviolet |
| 128. | V | Volume |
| 129. | $V_B$ | Breakthrough volume |
| 130. | $V_f$ | Volume of coating |
| 131. | $V_m$ | Volume of aqueous phase |
| 132. | $V_M$ | Hold-up or dead volume of the bed |
| 133. | $V_{PDMS}$ | Volume of PDMS |
| 134. | $V_s$ | Volume of sample |
| 135. | w | Extraction efficiency |
| 136. | β | Phase ratio |
| 137. | λ | Wavelength |

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# CHAPTER 1

# Background

## 1.1 Introduction

Drugs, depending upon the point of use and their concentration have beneficial as well as detrimental effects. A variety of analytical instrumentation has been employed for qualitative and quantitative analysis of drugs in countless circumstances. These include clinical control for diagnosis and treatment of diseases, doping control to monitor the use of drugs that stimulate the build-up of muscles mass as well as forensic toxicology to test for abuse of illegal drugs [1]. In addition, the widespread use of pesticides in agriculture and of veterinary drugs with therapeutic or growth promoting effects in zootechnics is a cause for concern. The effects of indirect exposure to food contaminated with drug residues have raised the demand for screenings of xenobiotics in foodstuffs before marketing [2]. Despite advances in sensitivity of analytical instrumentation for the end point determination of analytes in the environmental and other domains, sample handling is usually required to extract and isolate analytes of interest from complex matrices [3].

The objectives of this thesis were to evaluate and optimise the application of sample handling techniques based on solid phase extraction (SPE). Mixed-mode polymeric SPE sorbents were assessed for the simultaneous extraction of drugs with a diverse polarity and acidic, basic or neutral functionalities from biological matrices. In addition, the QuEChERS (***Qu****ick,* **E***asy,* **Ch***eap,* **E***ffective,* **R***ugged, and* **S***afe*) method was assessed for the analysis of drug residues from food and environmental matrices.

## 1.2 Sample Handling

Sample handling refers to any action applied to the sample before the analytical procedure [4]. Sample handling incorporates a number of processes that include: sampling and sample preparation (e.g. sample pretreatment, extraction, clean up and sample enrichment). Thorough sample handling is very important; it ensures the integrity of samples as well as prevents deterioration and cross contamination. Furthermore, it helps in maintaining sample tracking and in safety measures [5].

Sampling and sample preparation generally account for about 80% of the whole analysis time [6].

### 1.2.1 Sampling

Sampling is a process of collecting small portions (samples) that are representative of the whole population. By sampling only a fraction of the population, quality estimates can be obtained accurately, quickly, with less expense and time than if the whole population were measured. Since virtually no food material can be analyzed in its entirety, careful sampling techniques are required to obtain representative, laboratory-sized primary samples, in addition to subsequent subsamples, or secondary samples [7].

The size of the sample selected for analysis largely depends on the expected variations in properties within a population, the seriousness of the outcome if a bad sample is not detected, the cost of analysis and the type of analytical technique used. Based on this information it is often possible to use statistical techniques to design a sampling plan that specifies the minimum number of sub-samples that need to be analyzed to obtain an accurate representation of the population [8].

Furthermore, a checklist is usually devised as a guideline for carrying out an effective sampling strategy. The following checklist (see Table 1.2.1) was designed by Janusz Pawliszyn for drug analysis [9].

***Table 1.2.1: Checklist in carrying out an effective sampling strategy for drug analysis [9]***

| | Checklist: |
|---|---|
| 1. | **What are the data quality objectives?** |
| 2. | **Is specialized sampling equipment needed and/or available?** |
| 3. | **Are the samplers experienced in the type of sampling required /available?** |
| 4. | **Have all the analytes been listed? Have the detection level and methods been specified for each analyte?** |
| 5. | **Are the entire analytes stable in the sample? Is a special preservation method needed after the sampling?** |
| 6. | **Are there specific types of quality control samples? Does the instrument require optimization of its operating parameters?** |
| 7. | **What type of sampling approach will be used? Random, systematic, judgmental or a combination of these?** |
| 8. | **Will the type of sampling meet the data quality objectives? Is the sampling approach compatible with the data analysis method?** |
| 9. | **How many samples are needed? How many methods are specified? How many test samples are needed for each method?** |
| 10. | **What types of quality control samples are needed? How many exploratory samples are needed? How many supplementary samples will be taken?** |

### 1.2.2 Sample preparation

Sample preparation brings about the extraction of chemical residues from a sample with subsequent purification of the extract. Additionally, the residues of interest are isolated and any matrix interferences that may affect the detection system removed. Even with the advancement of separation and detection techniques, sample preparation is still a vital part of the analytical process. An effective sample preparation is essential to achieve reliable results and maintain instrument performance. Use of ideally cleaned samples also reduces the time to maintain instruments and in turn the cost of assay [10].

Sample preparation impacts nearly all the assayed steps and is hence critical for unequivocal identification, confirmation and quantification of analytes. Generally, a clean sample assists to improve separation and detection, while a poorly treated sample may invalidate the whole assay [11].

Sample preparation and sampling entail a series of unit operations, each operation/process capable of a specific task. These processes are the fundamental building blocks for any analytical procedure that can be matched to an analytical challenge at hand [12]. Lists of typical operations that are usually employed for sample handling are given in Fig. 1.2.1.

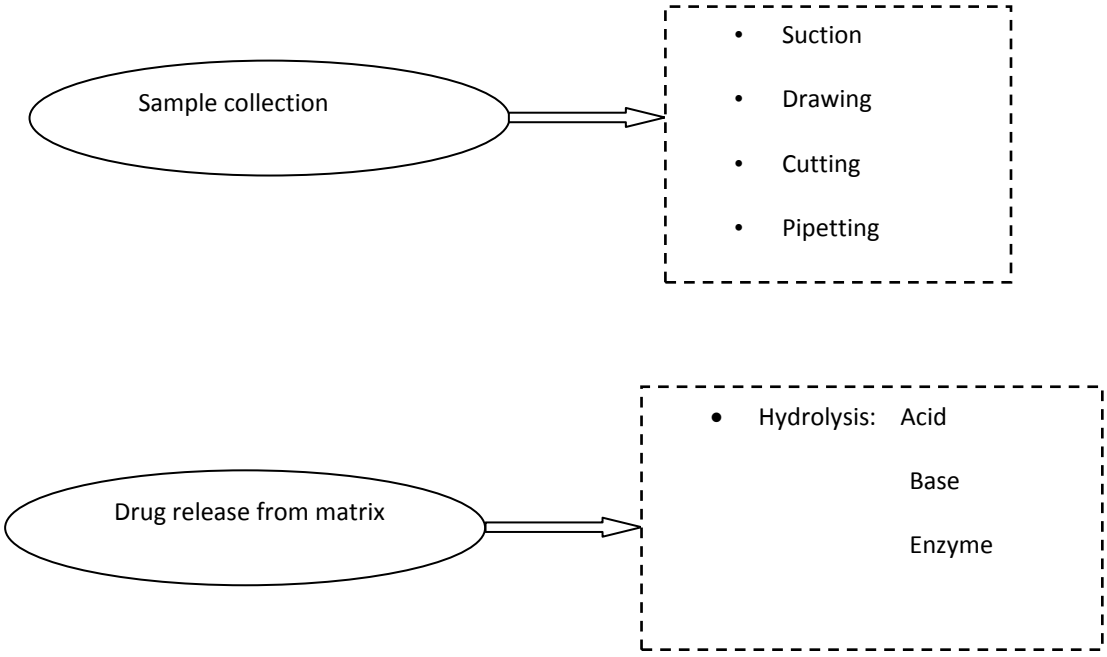
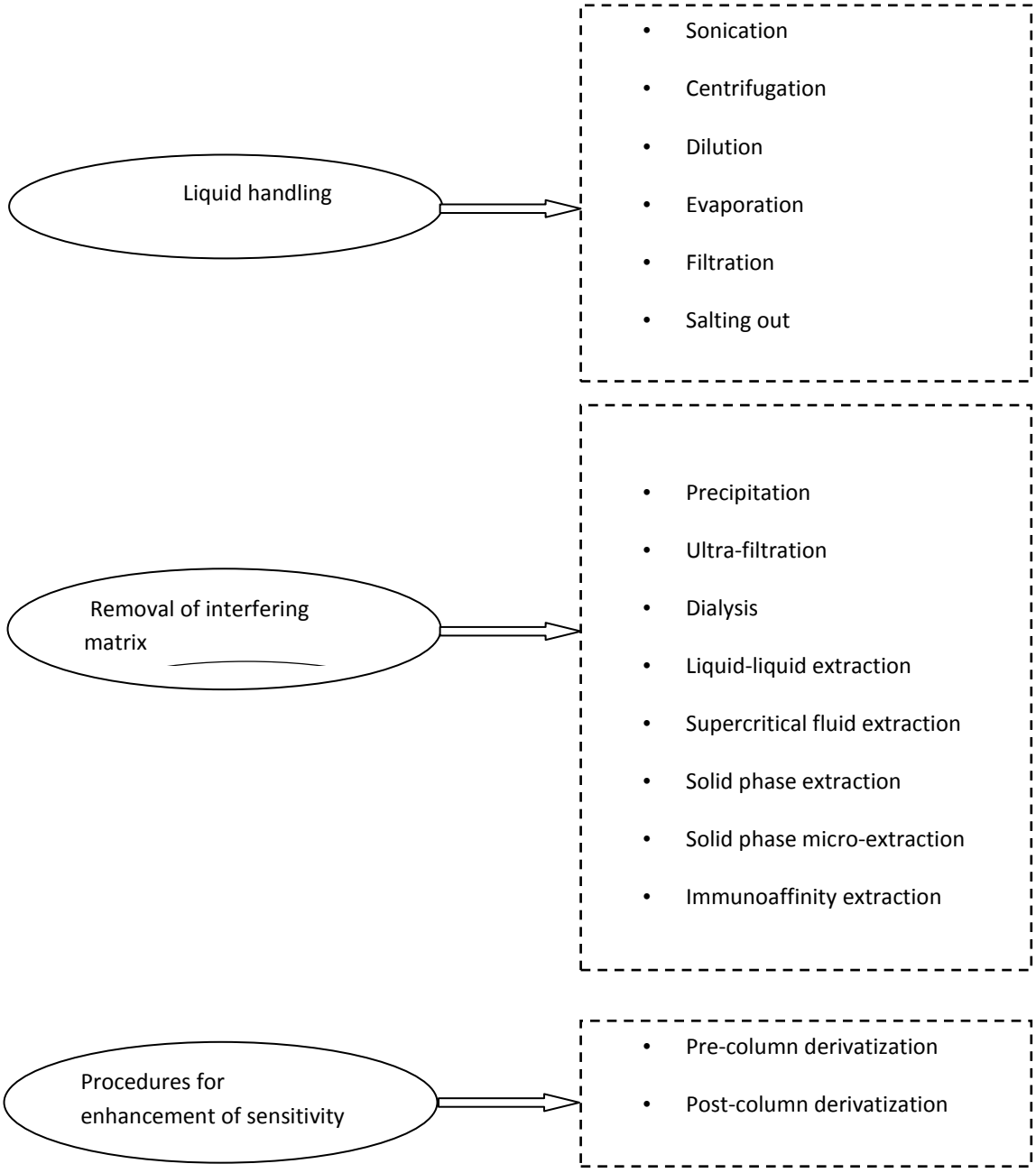



**Figure 1.2.1: Classification of Sampling and Sample Preparation unit operations [12]**




**…continuation of Fig. 1.2.1 [12]**

### *1.2.2.1 Drug release from matrix*

Drugs that have been conjugated typically with proteins, glucuronides or sulphate moiety need to be released or be made available to the assay [13 - 14]. Biological matrices are usually hydrolysed to release the drugs. Hydrolysis can be performed either by enzymes, acids or bases. Acidic or basic hydrolysis usually presents harsher conditions i.e. extremes of both pH and temperature can be encountered but they take less time and give cleaner extracts relative to enzymatic hydrolysis [15 - 16]. Food matrices are at times subjected to hydrolysis by enzymes (e.g. protease) [17].

### *1.2.2.2 Liquid handling Procedures*

The liquid handling procedures provide a link amongst the sample preparation operation units. Procedures for liquid handling include addition, mixing, removal or transfer of liquids. In both research and routine laboratory work the reliable measurement and dispensing of samples and reagents, which usually employs pipetting, is essential for the success of a quantitative analysis. Traditionally, pipetting has been done almost exclusively by suction using glass pipettes. Single- and multichannel mechanical pipettors and disposable tips were developed by the end of 1960s [18]. The observation was that mechanical air-displacement pipettors belong to the standard equipment of all laboratories and that new demands on more convenient and accurate pipetting devices have resulted in the development of electronic pipettors.

For drug residue analysis concentrations are in trace amount and a small error in pipetting can therefore cause a large error in the final result. The following are pointers on pipetting, as a precaution to minimize error [19]:

- The pipettor/tip should be chosen in a manner that will allow minimal air space between the piston and the liquid.
- The tip should not be placed too deep, but just under the surface of the liquid in the reservoir (2-3 mm).
- Pre-wetting the tip improves both accuracy and precision.
- The pipettor should be held vertically, not at angle.
- The aspiration should be done slowly.

Liquid handling is labor-intensive and often a rate-limiting step for sample preparation [9].

#### *1.2.2.3 Removal of interfering matrix components*

The analysis of complex matrices (e.g. in environmental, pharmaceutical, biochemical, organic chemistry and food industries) requires sample handling steps aimed at the removal of unwanted matrix constituents from the sample. For the increasingly sensitive chromatographic analyses good sample preparation is essential, because it protects the chromatographic columns and it allows a greater sensitivity by removal of interfering matrix components. A selective and specific sample preparation thus is a prerequisite for reasonable, economical and sensitive analyses [20].

A number of approaches to the removal of matrix interference have been reported. For instance, solid-phase extraction (SPE) is the predominant clean-up technique. However, the high matrix load of complex biofluids affects the efficiency of this extraction technique and gives rise to co-elution of interfering substances. This is particularly true for proteins, because many commercially available SPE sorbents are not biocompatible and cause non-specific adsorption and/or precipitation of proteins. With on-line SPE this, in turn, causes a clogging of the SPE column and shortens its lifetime dramatically. As a result, most sample clean-up procedures include a protein precipitation step in order to prevent these effects [21].

Examples of some of the approaches that have been employed for the removal of interfering matrix components include; solid phase micro-extraction (SPME) [22, 23]; liquid–liquid–liquid microextraction (LLLME) [24]; stir-bar sorptive extraction (SBSE) [25]; supported liquid membrane extraction (SLME) [26]; supercritical fluid extraction (SFE) [27]; QuEChERS [28 – 37] and microdialysis [38 – 39].

### *1.2.2.4 Procedures for enhancement of sensitivity and selectivity*

In LC analyses, UV chromophores and fluorophores are often introduced into sample molecules to increase their sensitivity to UV absorption and fluorescence detection respectively. Benzoyl chloride, m-toluol chloride and p-nitrobenzoyl chloride are reagents that can add a benzene ring to a solute molecule and render it UV absorbing. To introduce UV chromophores into a solute containing a carbonyl group, 3, 5-dinitrophenylhydrazine and p-nitrobenzylhydroxylamine are probably the two most common and effective reagents. To prepare fluorescent derivatives of phenols, and primary and secondary amines, dansyl chloride (5-dimethyl aminonaphthalene-1-sulphonyl chloride) is strongly recommended. Another fluorescent derivative is 4-chloro-7-nitrobenz-2,1,3-oxadiazole (NBD chloride) which provides highly fluorescent derivatives of primary and secondary amines but aromatic amines, phenols and thiols only yield weakly or non fluorescent derivatives [40].

For GC, non-volatile substances may be volatilized to render them amenable to detection. Silylating reagents are commonly used for GC derivatization. They have different functional groups (hydroxyl, carboxyl, amidic and amino groups) which render them versatile as derivatizing reagents [41 – 42].

# CHAPTER 2

# Sample Preparation Techniques

## 2.1 Overview

The extraction of analytes is based on differences in their chemical and physical properties. These typically include molecular weight, charge, solubility (hydrophobicity), polarity, or differences in volatility. Some extraction methods, such as immunoaffinity and imprinted polymers, utilise selectivity for specific structural groupings or mimic a biological selectivity. Furthermore, extraction of analytes is influenced by the penetration of solvent into the sample (mass transfer) and matrix effects. Solid samples are usually prepared by grinding directly or after drying, followed by solvent or liquid extraction. Organic or aqueous solvents are used to extract the analyte of interest, mostly followed by concentration or additional clean-up. These extract solutions can then be treated as liquid samples. Liquid samples can be handled directly by solvent–solvent extraction methods or sorption methods [43].

The basis of extraction procedures is described by the Nernst Distribution law as shown by Eqn. 2.1 [44].

$$K = \frac{C_1}{C_2} \qquad (2.1)$$

$$w = 1 - \left[\frac{1}{1 + K * V}\right]n \qquad (2.2)$$

Where; K = Nernst Distribution, $C_1/C_2$ = ratio of concentration of analyte in the upper phase/ concentration of the analyte lower phase, w = Extraction efficiency, V = ratio of volume in the upper phase/ volume in the lower phase, n = Number of cycle.

At constant temperature, a solute distributes itself between two immiscible phases such that the ratio of its concentrations in the two phases is constant. The distribution ratio between the two phases is influenced by: choice of the extracting solvent, pH value of the aqueous phase and the ratio of the volumes of the organic to aqueous phases. The extraction efficiency (2.2) is deduced from Eq. (2.1).

This chapter presents some of the sample preparation techniques employed for drug analysis and they include Soxhlet, supercritical fluid extraction, liquid-liquid extraction, solid-phase extraction based techniques (restricted access material, immunoaffinity extraction and molecularly imprinted polymers) and sorptive extraction (solid phase micro-extraction and stir bar sorptive extraction).

## 2.2 Soxhlet

In 1879 Franz Soxhlet assembled a set of extraction apparatus that was named after him, to separate lipids from solid samples [45]. Many applications of Soxhlet extraction are for environmental samples, such as soils, but it has been used for analysis of food particularly fat content or as a preliminary extraction technique for fat soluble analytes followed by further clean-up [46, 47]. It has also been used for the extraction of antioxidants from herbs and spices [48]. The Soxhlet equipment stimulated a great deal of interest because lengthy extractions could be performed unattended. Since then, the extraction of the compounds of interest into a suitable organic solvent has gained popularity in food and environmental analysis. In addition, there is no filtration, the extraction temperature is elevated hence the sample is repeatedly brought into contact with fresh solvent. Both polar and non-polar solvents can be used. The disadvantages of this technique are that it requires large quantities of solvent (300–500 ml). Furthermore, the solvent must be evaporated to concentrate analytes before determination. Thermally labile compounds can degrade due to elevated temperatures involved. The other limitation is that Soxhlet extraction is a single sample run that takes several hours or days to complete [49].

Most of the modifications have been aimed at bringing Soxhlet closer to that of the more recent techniques for solid sample preparation by shortening leaching times with the use of auxiliary energies and automating the extraction assembly [50]. High pressure, ultrasound or microwaves have been employed to minimise the negative characteristics of the conventional extractor (see Fig. 2.2.1). Further, automation of Soxhlet opened the door to commercialization of a number of different approaches such as the microwave assisted Soxhlet extraction.

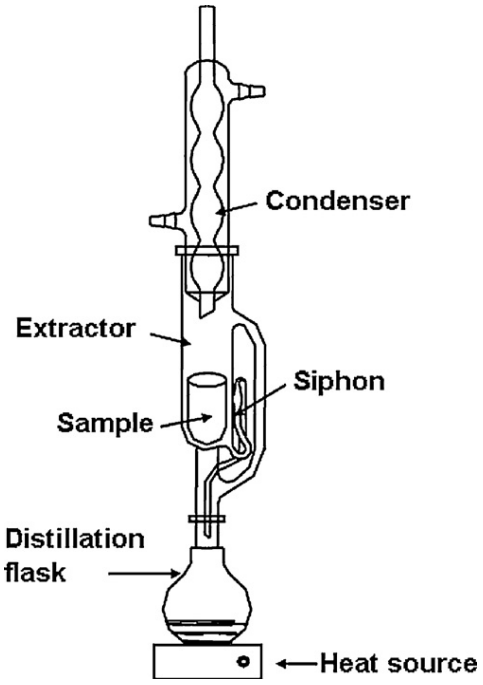



**Figure 2.2.1: Conventional Soxhlet extractor [49]**

Supercritical fluid extraction is one of the techniques that have emerged as an alternative to the traditional Soxhlet method.

## 2.3 Supercritical fluid extraction (SFE)

SFE resembles Soxhlet extraction, but the solvent used is a supercritical fluid, a substance above its critical temperature and pressure, which provides an unusual combination of properties. Supercritical fluids diffuse through solids similarly to gases, but dissolve analytes comparably to liquids. The extraction rate is enhanced and less thermal degradation occurs [51, 52]. In addition, sample handling can be done with non-polluting, non-toxic supercritical fluids, such as carbon dioxide, which is an excellent alternative to the potentially hazardous and expensive solvents used in Soxhlet extraction.

The high rate of penetration of the supercritical fluid in solid samples, such as food, even if slightly porous, permits fast back-diffusion of analytes, reducing extraction time. The complete step is performed in less than 20 min instead of several hours as required in traditional liquid-solid extraction. The technique can also be applied to thermally unstable analytes when using supercritical fluids with low critical temperature. One of the most interesting properties of these fluids is the direct relationship of solvent strength to density. Since the density of the fluid is a function of its temperature and pressure, precise control of these parameters allows a solvent with a narrow window of solvating strength to be obtained. It is possible, therefore, to substitute a variety of conventional solvents with a single supercritical fluid [53]. Supercritical fluid extraction equipment is fairly simple, as outlined in Fig. 2.3.1.

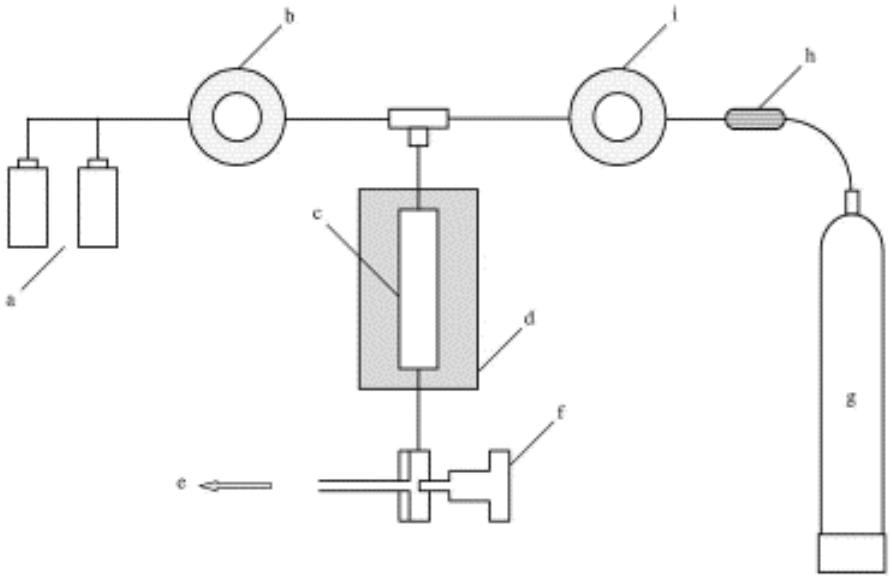



**Fig. 2.3.1: Schematic of SFE equipment: (a) modifier supply, (b) pump, (c) extraction cell, (d) furnace, (e) to collection, (f) flow restrictor, (g) fluid supply, (h) filter and (i) dual high-pressure piston pump [53].**

Selectivity and sample enrichment capabilities are limited for most of the solid sample techniques such as Soxhlet and SFE and usually require further clean-up and/or concentration steps for the determination of trace analytes. Extraction methods for liquid samples are based on partitioning into an immiscible extracting phase and can be used for a further clean-up of sample extracts obtained from the extraction of solids [54]. Liquid-liquid extraction is one of the solvent extraction techniques that maybe employed for a further clean-up.

## 2.4 Liquid-liquid extraction

Liquid–liquid extraction is based on the relative solubility of an analyte in two immiscible phases and is governed by the equilibrium distribution/partition coefficient (see Eqn. 2.3). Extraction of an analyte is achieved by the differences in solubilising power (polarity) of the two immiscible liquid phases. Liquid–liquid extraction is traditionally one of the most common methods of extraction, particularly for organic compounds from aqueous matrices [55].

The behavioral pattern of two immiscible solvents, say 'a' and 'b', is essentially non-ideal with respect to one another. If a third substance is made to dissolve in a two-phase mixture of the solvents (i.e., 'a' and 'b'), it may behave ideally in either phase provided its concentration in each individual phase is approximately small. Therefore, the ratio of the mole fractions of the solute in the two respective immiscible phases ('a' and 'b') will be a constant which is absolutely independent of the quality of the solute present. The constant (K) is known as the distribution coefficient or the partition coefficient [56].

$$K = \frac{C_a}{C_b} \qquad (2.3)$$

Where; $C_a$ = concentration of solute in solvent 'a', $C_b$ = concentration of solute in solvent 'b'

The Partition Law offers the following two limitations:

(a) K is not thermodynamically rigorous i.e., it takes no cognizance of the activities of the different species. In other words, it is solely applicable to very dilute solutions in which case the ratio of the activities almost approaches unity, and

(b) It does not hold good when the distributing substances encounters association or distribution in either phases (i.e., 'a' and 'b')

Liquid–liquid extractions are usually accomplished with a separating funnel. The two liquids are placed in the separating funnel and shaken to increase the surface area between the phases. When the extraction is complete, the liquids are allowed to separate; with the denser phase settling to the bottom of the separating funnel [Fig. 2.4.1].

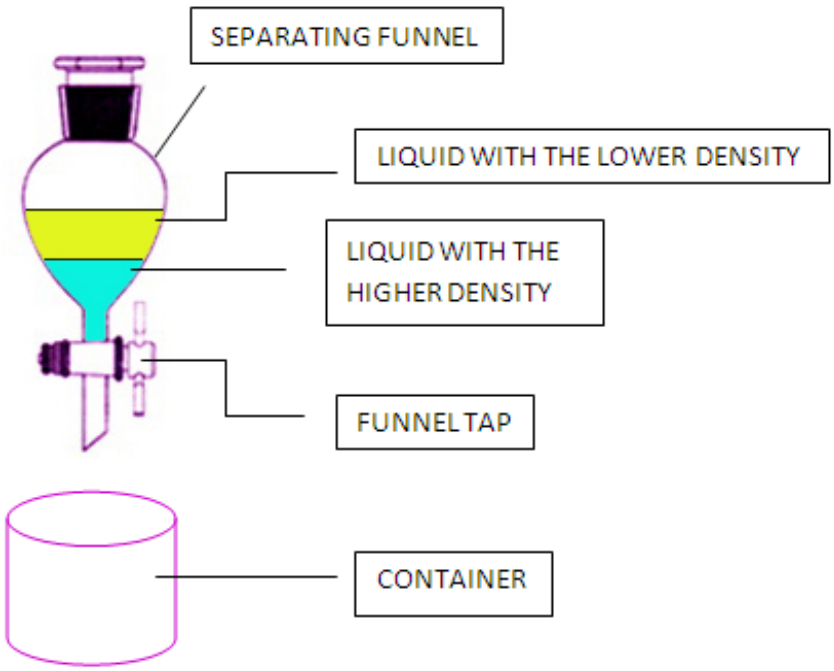



**Figure 2.4.1: Liquid-liquid extraction [57]**

The main advantage of LLE is the wide availability of pure solvents and the use of low cost apparatus. However, the technique suffers from some major drawbacks which include:

- Extracting with large amounts of toxic organic solvents results in the generation of vapors, which if not well vented to the atmosphere, could be hazardous.
- Additional clean-up steps are necessary.

- The technique is not suitable for highly polar compounds.
- The conditions used to evaporate the solvent may lead to low recovery of analytes due to degradation by heat, volatilization or adsorption to glass.
- The possible formation of emulsions when the immiscibility of the two phases is insufficient.
- The extraction and removing of waste solvent from a site are time consuming and expensive.
- The procedure is also not amenable to automation because several disjointed steps are usually required.

LLE is generally time consuming and labor intensive. Furthermore, LLE requires careful monitoring of extraction conditions such as temperature, pH and ionic strength. Due to these short comings, LLE tends to be replaced by solid phase extraction (SPE). SPE is attractive as it reduces consumption of and exposure to solvents, their disposal costs and extraction time [58].

## 2.5 Solid Phase Extraction

The principle of SPE is similar to that of LLE as it also involves partitioning of solutes between two phases. However, instead of two immiscible liquid phases, as in LLE, SPE involves partitioning between a liquid (sample matrix or solvent with analytes) and a solid phase (sorbent) [59]. SPE enables the concentration and purification of analytes from solution by sorption on a solid sorbent. The basic approach involves passing the liquid sample through a column, a cartridge, a tube or a disk containing an adsorbent that retains the analytes. After the entire sample has been passed through the sorbent, retained analytes are subsequently recovered upon elution with an appropriate solvent [60].

**Basic SPE principles**

An SPE technique consists of four to five successive steps (see Fig 2.5.1). First, the solid sorbent should be conditioned using an appropriate solvent, followed by the same solvent as the sample solvent (equilibration). This step is crucial, as it enables the wetting of the packing material and the solvation of the functional groups. In addition, it removes possible impurities initially contained in the sorbent or the packaging. Furthermore, the equilibration step removes the air present in the column and fills the void volume with solvent. The nature of the conditioning solvent depends on the solid sorbent. Typically, for reversed phase sorbent (such as octadecyl bonded silica), methanol is frequently used, followed with water or aqueous buffer whose pH and ionic strength are similar to that of the sample. Care must be taken not to allow the solid sorbent to dry between the conditioning and the sample treatment steps, otherwise the analytes will not be efficiently retained and poor recoveries will be obtained. If the sorbent dries for more than several minutes, it must be reconditioned [61].

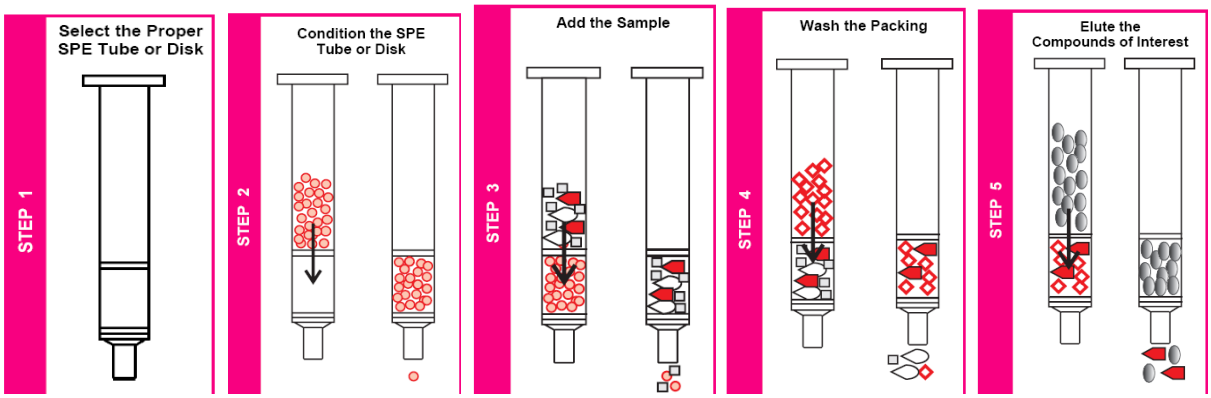
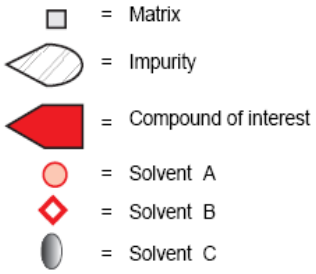



**Figure 2.5.1: SPE process [62]**

**Key to the process**




The second step is the percolation of the sample through the solid sorbent. Depending on the system used, volumes can range from 1 ml to 1 l. The sample may be applied to the column by gravity, pumping, aspirated by vacuum or by an automated system. The sample flow-rate through the sorbent should be low enough to enable efficient retention of the analytes, and high enough to avoid excessive duration. During this step, the analytes are concentrated on the sorbent. Even though matrix components may also be retained by the solid sorbent, some of them pass through, thus enabling some purification (matrix separation) of the sample [62].

The third step entails washing of the solid sorbent with an appropriate solvent having low elution strength. The washing step eliminates matrix components that have been retained by the solid sorbent, without displacing the analytes. A drying step may also be necessary, especially for aqueous matrices, to remove traces of water from the solid sorbent. This will eliminate the presence of water in the final extract. Water, in some cases, may hinder the subsequent concentration of the extract and/or the analysis. Pure solvents or mixtures of solvents differing significantly in polarity from the final eluent maybe useful wash solutions (see Table 2.5.1).

The final step is the elution of the analytes of interest by an appropriate solvent, without removing retained matrix components. The solvent volume should be adjusted so that quantitative recovery of the analytes is achieved with subsequent low dilution. In addition, the flow-rate should be correctly adjusted to ensure efficient elution. It is often recommended that the solvent volume be fractionated into two aliquots, one aliquot before the elution to let the solvent soak the solid sorbent and the other to elute the analytes [63].

**Table 2.5.1: Characteristics of solvents commonly used in SPE [62]**

| Polarity | | | Solvent | Miscible in water |
|---|---|---|---|---|
| **Non-polar** | Strong Reversed Phase | Weak Normal Phase | Hexane | No |
| | | | Isooctane | No |
| | | | Carbon tetrachloride | No |
| | | | Chloroform | No |
| | | | Dichloromethane | No |
| | | | Tetrahydrofuran | Yes |
| | | | Diethyl ether | No |
| | | | Ethyl acetate | Poorly |
| | | | Acetone | Yes |
| | | | Acetonitrile | Yes |
| | | | Isopropanol | Yes |
| | | | Methanol | Yes |
| | Weak Reversed Phase | Strong Normal Phase | Water | Yes |
| **Polar** | | | Acetic acid | Yes |

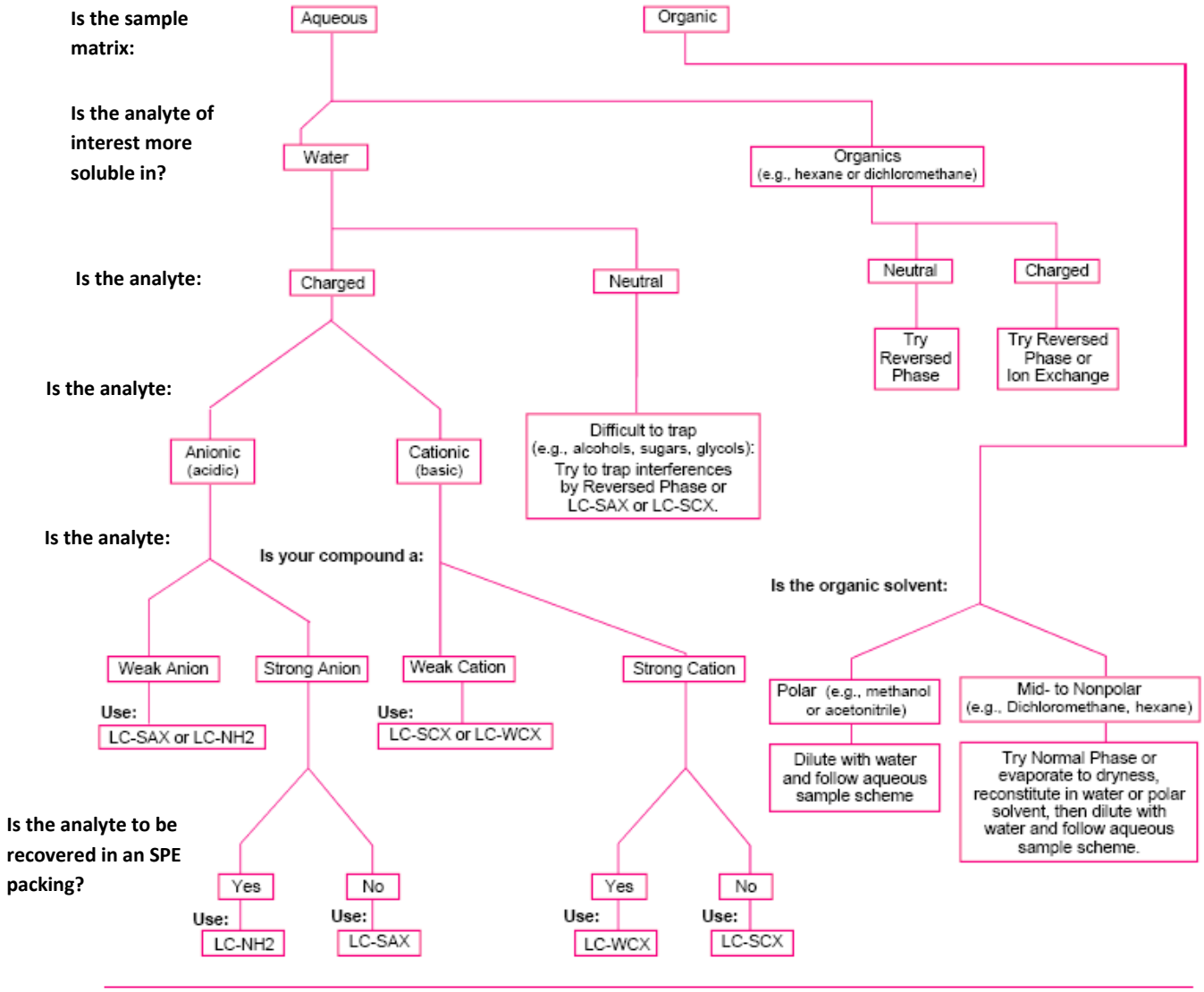
### 2.5.1 SPE Mechanisms

The selection of an appropriate SPE extraction sorbent is based on the binding interactions (retention mechanisms) between the sorbent and analyte of interest. Binding interactions include van der Waals forces (non-polar interactions), hydrogen bonding, dipole-dipole forces (polar interactions) and cation-anion interactions (ionic interactions) [64]. The energies associated with these binding forces vary considerably as shown in Table 2.5.2.

**Table 2.5.2: Energy associated with intermolecular forces [63]**

| Interaction Type | Energy (kJ/mol) |
|---|---|
| ***Hydrophobic interaction*** | |
| **van der Waals forces** | 1 – 5 |
| ***Polar Interactions*** | |
| **Dipole induced dipole** | 2 – 7 |
| **Dipole - dipole** | 5 – 10 |
| **Hydrogen bonding** | 10 – 25 |
| **Ion – dipole** | 10 - 50 |
| ***Ionic Interactions*** | |
| **Electrostatic (ion-ion)** | 50 - 500 |
| **Covalent bonding** | 200 - 1000 |

Selecting a mechanism for determining an SPE process is, in addition, based on considerations summarized in Fig. 2.5.1 below.




**Scheme 2.5.1: Method guide for selection of SPE procedure [62]**

### 2.5.2 SPE Sorbents

The physicochemical and thermodynamic dependencies between sorbents, analytes and solvents are used to optimise an SPE process during method development. The physical characteristics of the sorbent include: surface area, particle size, pore size or pore volume. Furthermore, the extraction ability of sorbents also depends on the bed capacity; the volume of sample loaded on the bed, the nature and volumes of conditioning solvents and eluents. The following are some SPE parameters to consider during method development: breakthrough volume, volume of rinsing solvent, elution volume, which depends on the kinetic properties of SPE bed, its hold-up volume and retention factor [65].

The breakthrough volume ($V_B$) is one of the parameters characterizing the sorbent bed. $V_B$ is defined as the sample volume which can be loaded on the sorbent bed without the loss of the analytes. $V_B$ depends on the concentration of the loaded analytes, becoming independent at lower concentrations, temperature, flow-rate and number of theoretical plates, a point which is often overlooked in experimental studies. $V_B$ can be experimentally determined by either on-line or off-line methods, however, off-line methods are time-consuming and somewhat subjective. A relationship between the breakthrough volume and the properties of the SPE devices: *N*, number of plates, $V_M$, hold-up or dead volume of the bed and *k*, retention factor of the analyte can be deduced from the theory of frontal chromatography. Eqn. (2.4) is applicable to systems with a large plate number [66].

$$V_B = (1+k)V_m\left(1-\frac{2.3}{\sqrt{N}}\right) \qquad (2.4)$$

There are basically five principle separation modes available for SPE sorbent chemistries (see Table 2.5.3).

**Table 2.5.3: Principle SPE separation modes [67]**

| Mode | Sorbent | Sample matrix |
|---|---|---|
| Non-polar | $C_{18}$, $C_8$, $C_6$,$C_4$,$C_2$, PH, CH, CN | Aqueous/ moderately polar |
| Polar | CN, Si, $NH_2$, Diol | Non-polar |
| Cation-exchange | SCX, PRS, CBA | Aqueous |
| Anion-exchange | SAX, $NH_2$, PSA, DEA | Aqueous |
| Mixed-mode | $C_8$ + SCX, $C_8$ + SAX, HLB | Aqueous |

The common goals of sample clean-up processes are that it should: be effective and automated, perform a selective removal and quantitative depletion of undesired, i.e., highly abundant constituents, selectively enrich the target compounds, significantly reduce the complexity of the whole process and enhance the reproducibility, repeatability, robustness and reliability of the method. Sample clean-up procedures using restricted access material (RAM) bear a high potential to fulfill most of these challenges [54].

### *2.5.2.1 Restricted Access Material (RAM)*

Mixed-mode sorbents incorporate the clean-up of sample in the SPE process, but the sequence is typically an off-line procedure and the deprotonation of biological samples (e.g. plasma and serum) is usually required before extraction. Moreover, there is an interest in employing on-line techniques for the handling of untreated biological samples [68]. Restricted access materials (RAMs) enable direct injection of the biological sample into flow-analysis systems without previous sample treatment.

RAMs are porous chromatographic supports specifically designed for the removal of macromolecules, partially based on a size-exclusion mechanism. Only small molecules are able to penetrate into the pores and interact with a stationary phase bonded on the inner surface, while large molecules are eluted with the clean-up mobile phase [69]. Once this fractionation is achieved, the elution of the analytes is performed with another mobile phase composition. RAMs were initially designed to remove proteins in the analysis of drugs in biological matrices such as plasma or urine. They have also found applications in environmental analysis, basically for the removal of humic substances while applications to food analysis are still scarce [70].

The first RAM support for the direct injection of biological matrices was named the internal surface reversed-phase (ISRP) [71]. It entails porous silica particles with the outer surface covered by a hydrophilic moiety limiting the adsorption of protein (diol-glycine groups) with a hydrophobic tripeptide partitioning phase (glycine-L -phenylalanine-L -phenylalanine or GFF) only on the internal surface. A schematic drawing of GFF material is presented in Fig. 2.5.2.1. The retention mechanism is mainly due to π-electron interactions [72].

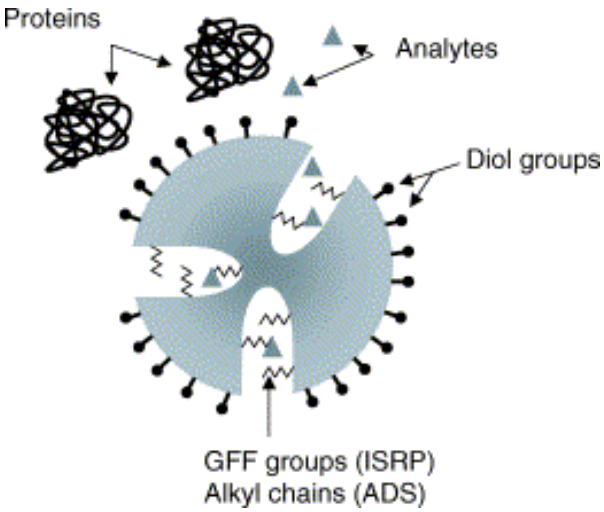



**Figure 2.5.2.1: Internal surface reversed-phase (ISRP) with GFF groups and alkyl-diol-silica (ADS) with alkyl chains [72].**

There are five basic types of RAM, classified according to the nature of the barrier and surface structure of the sorbent [93] and they include:

- mixed-functional phases and dual-zone materials;
- internal surface reversed-phase packing;
- shielded hydrophobic phases;
- semi-permeable surfaces; and,
- polymeric materials.

Most of the polar organic compounds cannot be determined at trace-level by LC as they co-elution with humic and fulvic substances present in high amount. Evidence of these compounds is usually seen as an interfering matrix peak at the beginning of the chromatogram or a large hump in the first part of the chromatogram depending on the gradient shape. Additional clean-up procedures are usually required prior to the final chromatographic analysis.

There is an interest in having highly selective SPE sorbents that allow extraction, concentration and clean-up in a single step. That was achieved by using materials involving antigen–antibody interactions, thus providing selective extraction methods based on molecular recognition [82]. Antibodies are covalently bonded onto an appropriate sorbent to form an immunosorbent, which is packed into an SPE cartridge or precolumn. Since antibodies are highly selective towards the analyte they are able to initiate the immune response with a high affinity. The corresponding immunosorbent may then extract and isolate the analyte from complex matrices in a single step, and the problem of co-extraction of matrix interferences is therefore solved.

#### *2.5.2.2 Immunoaffinity Extraction*

Immunoaffinity extraction (IAE) is based upon a molecular recognition mechanism where the high affinity and high selectivity of the antigen–antibody interactions allow the specific extraction and the concentration of the analytes of interest in one step [74]. IEA can efficiently eliminate the matrix contaminations and non-target compounds to enrich the target analyte. Immunoaffinity extraction has been applied in environmental monitoring [75], pharmaceutical and biomedical analyses [76], and food analysis [77]. As a cleanup and separation technique, IAE has been successfully used to enrich analytes in biological fluid prior to CE detection [78]. The principle of IAE is demonstrated in Fig. 2.5.2.2. The immunosorbent is packed on either a disposable cartridge or an LC pre-column.

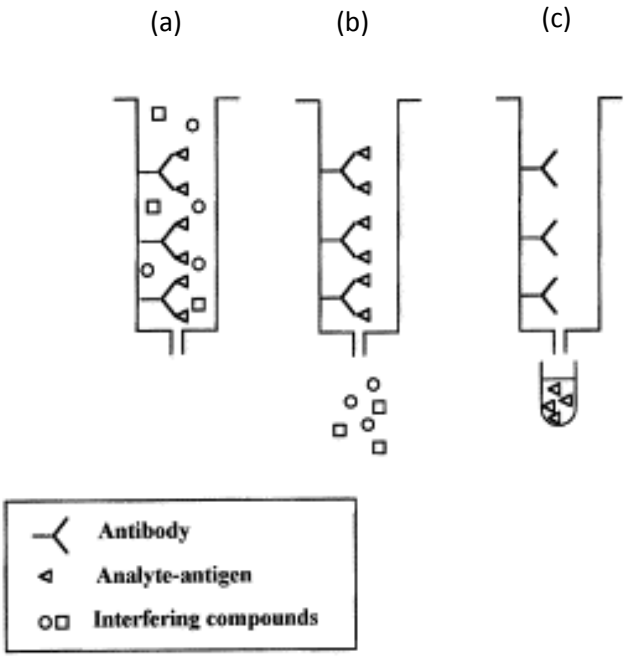



**Figure 2.5.2.2: Off-line procedure used for the immune-sample pretreatment on immunosorbents. (a) percolation of the sample; (b) washing to eliminate the non-retained compounds; (c) elution of compounds retained by the immobilized antibodies [75].**

One of the major disadvantages of IAE is that immunosorbents, based on molecular recognition by antibodies, show high selectivity to target molecules, but because they are less stable, difficult to prepare, and expensive, their applications are to some extent limited [78]. In addition, the analyte-antibody interaction can also be affected by the sample matrix, leading to low extraction recoveries. Rather than being dependent on antibody production, attempts have been made to mimic the specificity of immunological products with synthetic molecularly imprinted polymers [79].

### *2.5.2.3 Molecularly Imprinted Polymers (MIPs)*

MIPs are tailor-made materials with high selectivity for a target molecule. Generally, MIPs are synthesized by assembling monomers around a template to form a complex through covalent or non-covalent interactions and joined by a cross-linking agent (Fig. 2.5.2.3). Then the template molecule is removed by chemical reactions or extraction, resulting in exposure of binding sites ('imprints') which are complementary to the template in size, shape, and position of the functional groups, and consequently allows its selective uptake [80, 81].

***Imprinting techniques***

The most common approach to MIPs is non-covalent imprinting. In this process, the complex of template and functional monomer is formed *in situ* by non-covalent interactions, such as hydrogen bonding, electrostatic forces, van der Waals forces, or hydrophobic interactions. Moreover, the rebinding of template molecules with MIPs is also carried out by the same non-covalent interactions. The advantages of this technique include; easy preparation of the template/monomer complex, easy removal of the templates from the polymers, fast binding of templates to MIPs and its potential application to a wide range of target molecules.

Another preparation technique is the covalent imprinting. The complex is formed by covalent-linkage of a functional monomer and template prior to polymerization. After the removal of the template by chemical reaction, the MIPs obtained rebind template molecules via the same covalent interactions. The main advantages of this technique are that the monomer/template complexes are stable and that a wide variety of polymerization conditions can be employed. The limitation of covalent imprinting is the slow release and binding of templates.

The third technique is the hybridization of covalent and non-covalent imprinting, also called semi-covalent imprinting. In this process, the polymers are prepared like those in covalent imprinting, while the guest binding employs non-covalent interactions. Semi-covalent imprinting combines the main advantages of the above two techniques, the stability of the complex in covalent imprinting and the fast guest binding in non-covalent imprinting [82].

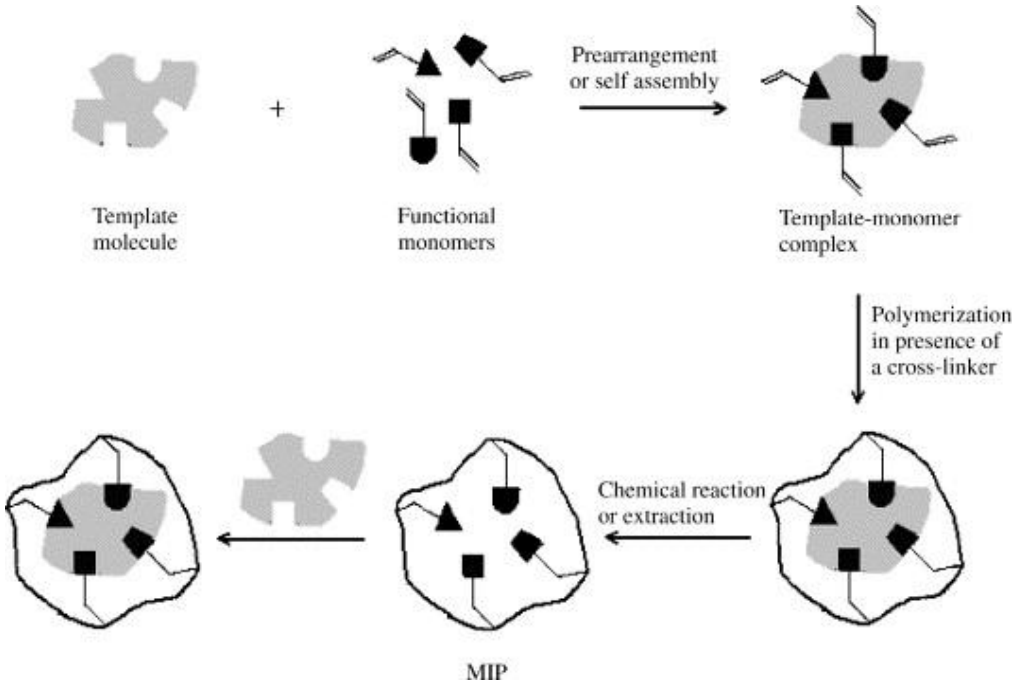



**Figure 2.5.2.3: Synthesis of MIPs and its selective recognition to target molecule [82]**

MIPs have been widely used as artificial receptors in separations, sensors, catalysis, chemical, pharmaceutical and biotechnological industries. They were employed for separation and purification of amino acids, DNA and RNA, peptides, hormones and carbohydrates, and for the recovery of flavor compounds. Furthermore MIPs were used in the environmental industry for the removal of pesticides, endocrine-disrupting compounds and heavy metals from waste and drinking water. Among these applications, is the common use for SPE, for which MIPs are commercialized [83].

In the light of green chemistry, innovations are towards solvent-free sample preparation for the extraction and enrichment of analytes of interest from aqueous matrices. Most sample-enrichment procedures employ adsorbent materials where good performances (e.g. high recoveries) are attained under many practical challenges. However, in some cases the applicability of adsorptive sample preparation falls short, especially for the enrichment of polar and/or high-molecular-weight compounds, particularly with thermal desorption. Furthermore, polar compounds readily undergo surface-catalyzed reactions and on desorption they yield compounds different from those originally sampled. The other challenge is that high-molecular-weight compounds cannot be desorbed because of extremely strong interactions with the adsorbent and their low volatility. Sample preparation based on sorption extraction has been developed over the years as a means to overcome some of these challenges [84]. Typical examples for sorptive extraction include solid phase micro-extraction (SPME) and stir bar sorptive extraction (SBSE).

### *2.5.3 Sorptive Extraction*

Sorptive extraction is based on the distribution equilibria between the sample matrix and a non-miscible liquid phase. Matrices are mostly aqueous and the non-miscible phase (e.g. polydimethylsiloxane, PDMS) is often coated onto a solid support. Analytes are 'extracted' from the matrix into the non-miscible 'extracting' phase. Unlike adsorption techniques (such as SPE), where the analytes are bound to active sites on the surface, the total volume of extraction phase is important. For SPME, the volume of polydimethylsiloxane is approximately 0.5 µl while 25- 125 µl polydimethylsiloxane coatings are used in stir-bar sorptive extraction. Extraction of analytes depends on the partitioning coefficient of solutes between the phases [85]. The octanol-water distribution coefficient ($K_{ow}$) can be used to demonstrate how well a given analyte will be extracted (see Fig.2.2.3).

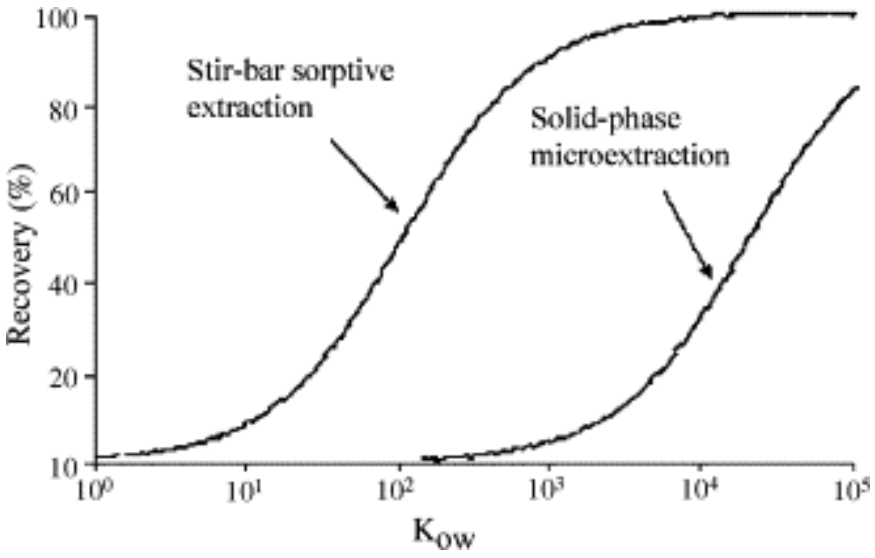



**Fig. 2.5.3: Theoretical recovery (%) in function of solute log Ko/w for SPME (100 µm fiber, 0.5 µl PDMS) and SBSE (1 cm × 0.5 mm df, 25 µl PDMS) and 10 mL sample volume. Equilibrium sampling is assumed [84].**

The partitioning coefficient has been correlated with the octanol–water distribution coefficients ($K_{o/w}$). However, the octanol–water distribution coefficient only gives a good indication if and how well a given solute can be extracted with SPME or SBSE. It is very important in this respect to realize that the sorptive equilibrium is also dependent upon the phase ratio and thus on the amount of polydimethylsiloxane applied [86]. This relationship is shown in Eq. (2.4).

$$K_{PDMS\ /\ w} = \beta \frac{m_{PDMS}}{m_w} \qquad (2.4)$$

$$\beta = \frac{V_m}{V_{PDMS}} \qquad (2.5)$$

Where; $K_{PDMS/w}$ = the distribution coefficient between PMDS and water, β = phase ratio (Eq. 2.5) $m_{PDMS}$ = mass of the solute in the PDMS phase, $m_w$ = mass of the solute in the aqueous phase, $V_{PDMS}$= volume of PDMS, $V_m$ = volume of aqueous phase.

Illustrated in this thesis are the different profiles that can be obtained using the SPME and SBSE.

### *2.5.3.1 Solid-phase micro-extraction (SPME)*

SPME is a solvent-free extraction method first introduced by Arthur and Pawliszyn in 1990 [9]. It was mainly applied for the extraction of volatile and semi-volatile organic pollutants in water samples and has since been extended to various samples which include biological matrices, for example, whole-blood, plasma, urine and hair [87] as well as food samples [88].

SPME uses a short piece of a fused-silica fibre coated with a polymeric stationary phase placed on a syringe (see Fig. 2.5.3.1). During transport, storage and manipulation, the fibre is retracted into the needle of the device. SPME is a two step process, firstly the partitioning of analytes between the sample matrix (can be a liquid sample or headspace vapour) and the fibre coating, and then desorption of the (concentrated) extract from the fibre into the analytical instrument, usually a GC, where the sample components are thermally desorbed. The fibre can also be extracted (desorbed) into an LC eluent using a static or dynamic mode and several commercial interfaces are available [89].

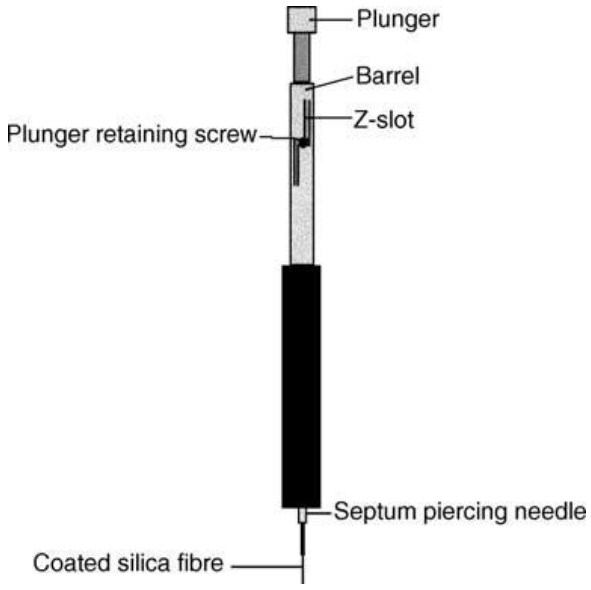



**Figure 2.5.3.1: Components of SPME device [90]**

The affinity of fibres for the analyte relies on the principle of 'like dissolves like', therefore, non-polar polydimethylsiloxane (PDMS) fibre will be preferred for the extraction of non-polar analytes, while the more polar polyacrilate (PA) fibre will be more appropriate for the extraction of polar analytes [91]. The amount of analyte extracted onto the fibre depends on the polarity and thickness of the stationary phase as well as on the extraction conditions and concentration of the analyte in the sample. Extraction of analyte is typically improved by agitation, addition of sodium chloride or other salt to the sample, changing the pH, and increasing the temperature [88].

At equilibrium the amount (n) of the analyte adsorbed by the fibre is related to the concentration of the analyte in the sample by the law of conservation of mass (see Eq. 2.6).

$$n = \frac{K_{fs} V_f C_o V_s}{K_{fs} V_f + V_s} \qquad (2.6)$$

Where; n = mass of the analyte adsorbed by the fibre coating, $C_o$ = initial concentration of the analyte in sample, $K_{fs}$ = partition coefficient for the analyte between fibre coating and the sample matrix, $V_f$ = volume of coating, $V_s$ = volume of sample.

The coatings used in SPME have strong affinities for the organic compounds they are intended to extract and therefore $K_{fs}$ values for these analytes are large. Consequently, SPME has a very effective concentrating factor that leads to good sensitivity [92]. However, in SPME the quantity of extraction medium (e.g., the capacity of polydimethylsiloxane coated on the fibre) is very limited. For a typical 100 μm polydimethylsiloxane fibre, the volume of extraction phase is approximately 0.5 μl. Furthermore, for very polar compounds, competition can occur between the aqueous phase, the SPME fibre, the glass wall of the extraction vessel, and the surface of the polytetrafluoroethylene stir bar used to stir samples. Based upon these observations, an extraction method based on stir bars was developed [93].

Stir bars were coated with a layer of PDMS and used to stir aqueous samples, thereby extracting and enriching solutes into the PDMS layer. The extraction phase in SBSE is the same as that in SPME, although its quantity is 50–250 times larger. After extraction, the solutes are thermally desorbed and analyzed by GC in a similar manner to SPME. Alternatively, the analytes can be eluted by LC. Therefore, the basic principles of SPME and SBSE are identical [94].

### *2.5.3.2 Stir bar sorptive extraction (SBSE)*

Stir bar sorptive extraction (SBSE) was introduced by Baltussen et al. in 1999 as a solvent-less sample preparation method for the extraction and enrichment of organic compounds from aqueous matrices [89]. The method is based on the same sorptive extraction principle as SPME whereby the solutes are extracted into a polymer coating on a magnetic stirring rod [95]. A suitable amount of sample is placed in a headspace vial or a container with a PDMS-coated stir bar and the sample is stirred for 30–240 min (Fig. 2.5.3.2).

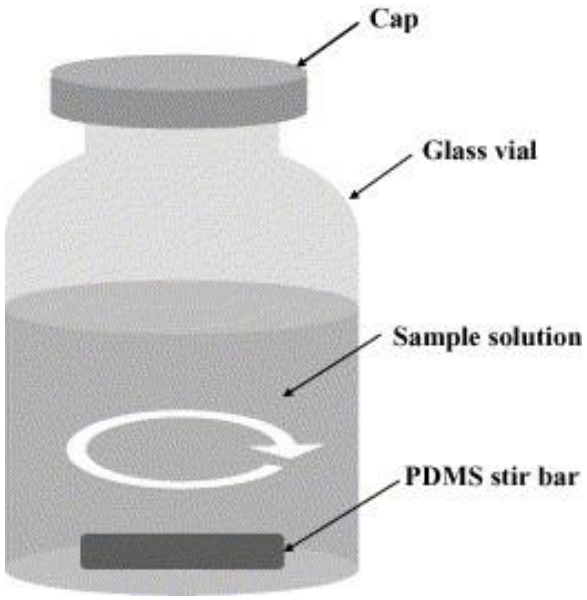



**Figure 2.5.3.2: SBSE set-up [95]**

SBSE consists of two major steps: extraction and desorption.

### *Extraction step*

During extraction the polymer-coated stir-bar is put in contact with the solutes by immersion or by headspace. In the immersion mode, which is usually abbreviated simply as SBSE, the polymer-coated stir-bar is added to a headspace vial that contains the liquid sample and the sample is stirred under controlled physical and chemical conditions. After extraction, the stir-bar is removed, rinsed with distilled water in order to remove salts, sugars, proteins or other sample components, dipped on a clean paper tissue to remove water, and submitted to desorption. The rinsing step is extremely important when analytes are thermally desorbed in order to avoid the formation of non-volatile material that can clog the desorption unit [96].

### *Desorption step*

Most SBSE applications involve the use of thermal desorption (TD) followed by GC to recover the analytes accumulated in the coated stir-bar which implies not using organic solvents and allows the complete introduction of the extracted solutes in the chromatographic system [97]. Liquid desorption (LD) is an alternative to TD for thermally labile analytes, particularly when the separation is carried out using liquid chromatography (LC) or capillary electrophoresis (CE). During LD mode, the polymer-coated stir-bar is immersed in a stripping solvent or solvent mixture for the chemical desorption of the extracted solutes. The minimum stripping solvent volume must guarantee the complete immersion of the coated stir-bar and, obviously, the solvents or mixtures used in this step must be compatible with the polymer. Acetonitrile (MeCN), methanol (MeOH), mixtures of these solvents or mixtures with water or aqueous buffers are the most common desorption solvents [98].

The extraction time is controlled kinetically; it is determined by the sample volume, the stirring speed and the stir bar dimensions, and must be optimized for a given application. Optimization is normally accomplished by measuring analyte recovery as a function of the extraction time. The optimum conditions are obtained when no additional recovery is observed even when the extraction time is increased further [95].

The percentage recovery (%*R*) of a given SBSE setup can be calculated as follows [98]:

$$\%R = \frac{K_{PDMS/w}}{K_{PDMS/w} + \beta} \times 100 \qquad (2.6)$$

Where; $K_{PDMS/w}$ = the distribution coefficient between PMDS and water, β = phase ratio (Eq. 2.4)

Although SBSE is widely applied in environmental, food and biomedical analysis, it has some limitations which include the fact that the coated stir bar cannot be directly desorbed in a simple split/splitless injection port of a gas chromatograph. Hence the analyte has to be back extracted into a fitting solvent, which adds an additional step to the overall analytical method. Another drawback is presented during extraction, as it takes long to reach equilibration time. Working under equilibrium guarantees maximum sensitivity and a better precision. However, sometimes, in order to minimize analysis time, sensitivity and precision are sacrificed by working under non-equilibrium conditions [98].

Two modern and environmentally friendly enrichment techniques, stir bar sorptive extraction and membrane-assisted solvent extraction (MASE) were compared for the determination of 18 organic contaminant residues in Brazilian sugarcane juice. Stir bar sorptive extraction and thermal desorption coupled to capillary gas chromatography-mass spectrometry using the selected ion monitoring mode [SBSE-TD-GC-MS(SIM)] and membrane-assisted solvent extraction combined with large volume injection [MASE-LVI-GC-MS(SIM)] methods were assessed taking into account the time of extraction [SBSE (3h) and MASE (30min)]. It was concluded that, faster analyses and much better analyte recovery results were achieved with MASE, whereas greater sensitivity and repeatability were obtained with SBSE [99]. MASE is carried out by using a non-porous membrane as interface between the sample and the organic solvent which prevents mixing the two phases and provides selectivity and specificity in terms of permeation and transport through the membrane.

A literature review on the application of some of the sample preparation techniques described will be presented in chapter 3 of this thesis. The review includes applications in environmental, food and biomedical fields in analyzing for drugs selected as representatives of acidic, basic or neutral drugs, polycyclic aromatic hydrocarbons, sulfonamides and acrylamide.

# CHAPTER 3

# Literature review on drug and organic pollutant residue analysis

## 3.1 Overview

This chapter presents background information on the analytes of interest in this thesis, with emphasis on the sample preparation aspect of the whole analytical protocol. The chapter is therefore divided into four sections:

3.2 Acidic, basic and neutral drugs in biological matrices.
3.3 Organic pollutants (PAHs) in food and environmental matrices.
3.4 Veterinary drugs residues (sulfonamides) in food matrices.
3.5 Acrylamide in food matrices.

## 3.2 Acidic, basic and neutral drugs in complex/biological matrices

In forensic toxicology, body fluids are monitored for therapeutic drugs that may have been abused and/or resulted in poisonings and death. One family of frequently monitored drugs that are characterized by acidic or neutral chemical properties is composed of non-opioid analgesics, anticonvulsants and barbiturates. Among the analgesics, paracetamol/acetaminophen is widely used as an over-the-counter drug for the reduction of pain and fever.

The analgesics naproxen, ketoprofen, etodolac, diclofenac and aspirin belong to the group of non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are generally used in the management of mild to moderate pain, fever and inflammation. While these are relatively safe drugs they may lead to severe toxic effects in the case of overdose or long term abuse [100]. The anticonvulsants (antiepileptic drugs, AEDs) are a heterogeneous group of substances that, among others include phenobarbital. These drugs are not usually abused, but they may impair the ability to drive a car and lead to accidental or suicidal poisonings. Barbiturates have been used extensively in the past to reduce anxiety, respiration, blood pressure, heart rate and rapid eye movement (REM). Sedative barbiturates compounds dispense into all tissue and organs in vivo, even cross the placenta barrier. However, barbiturates and their metabolites tend to accumulate in tissues. This accumulation could lead to tolerance, dependence, excessive sedation and cause anesthesia, coma and even death. Barbiturates have therefore been prohibited to men and to acting as animal feed additive and chemical protection drugs in animal. They could however, make animals (e.g. pig) drowsy and move-less, accelerate up-growth, and decrease feed cost. They are still being misused as animal feed additive and chemical protection drugs in animal butchery and in horse races. It is therefore necessary to monitor their residues to protect the consumer's health [101].

Chromatographic techniques such as high performance liquid chromatography with diode array detection (HPLC-DAD) are often used to monitor the levels of therapeutic drugs in biological fluids [102]. However, for some drugs (e.g., diclofenac), the therapeutic concentrations are relatively low, making HPLC-DAD unsuitable as an analytical tool without pre-concentration and clean-up of the analyte extract. Methods based on LC–MS/MS have been published for the determination of some barbiturates [103], anticonvulsants and analgesics [104] in serum, plasma and urine [105, 106]. Most of these methodologies employed extraction methods such as LLE and SPE however, LLE proved to be tedious and time consuming. Alternatively, SPE extraction methods though efficient and reliable, were not rapid enough for emergency toxicological screening and required the preparation of various buffers and solutions [107,108].

For lower detection limits, sample pretreatment based on methods, such as SPME [109] and SBSE [110] have been employed before HPLC analyses. SBSE and liquid desorption followed by high performance liquid chromatography with diode array detection (SBSE-LD -HPLC/DAD) was assessed for the simultaneous detection of several steroid sex hormones (SSHs) in water and urine matrices [111]. An extraction method based on solid phase micro-extraction membrane (SPMEM) was employed to extract tetrahydrocannabinol (THC) and cannabidiol (CBD) from blood and brain of the injected male mice as well as in spiked human urine. SPMEM is an extraction technique that integrates sampling, extraction and concentration into a single step. In addition SPMEM combines the advantages of both the SPME and membrane separation. The extracted THC and CBD were further determined with LC–MS. The reported method was found to be simpler and more convenient than the conventional liquid–liquid and solid phase extractions [112].

Mixed-mode ion exchange solid phase extraction has been utilized extensively in the pharmaceutical industry. It was employed for the isolation, purification and concentration of pharmaceuticals from interfering biological matrices. Several examples of the use of mixed-mode ion exchange solid phase extraction to illustrate the utility of this technique have been reported [113, 114].

For this thesis, ketoprofen, naproxen, phenobarbital and p-toluamide were selected to represent acidic drugs; amphetamine, m-touluidine and nortriptyline represented basic drugs while secobarbital and acetaminophen were neutral drug representatives see Tables 3.2.1 (a) and (b). The objective was to simultaneously fractionate acidic, basic or neutral drugs from urine and plasma with mixed-mode ion exchange polymeric resins, SampliQ-SAX and SampliQ SCX.

**Table 3.2.1 (a): Characteristics of acidic, basic and neutral drugs from urine matrix**

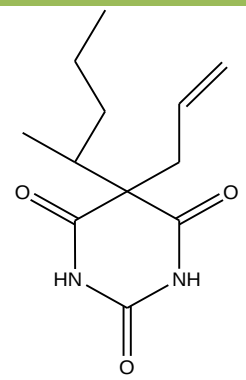
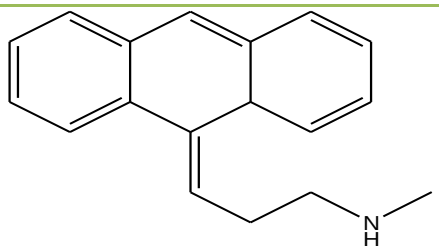
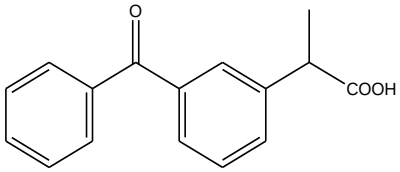
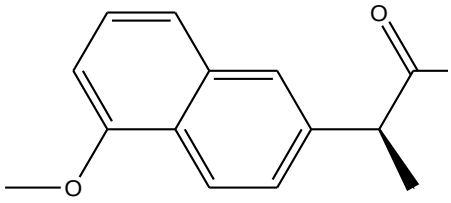
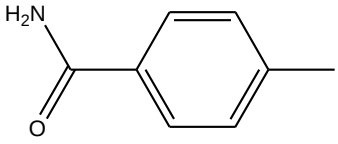
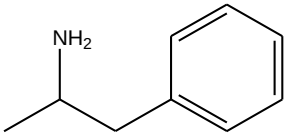
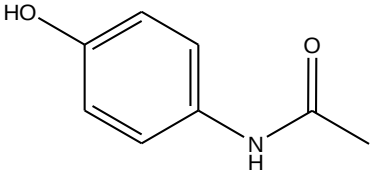
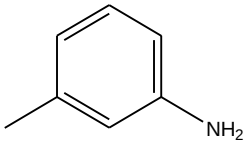
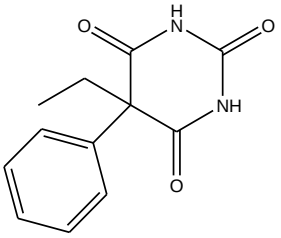
| Drug | Pharmacology | Classification | Structure | Log P | pKa |
|---|---|---|---|---|---|
| **Secobarbital** | Sedative | Neutral |  | 1.97 | 7.90 |
| **Nortriptyline** | Antidepressant | Basic |  | 4.28 | 9.70 |
| **Ketoprofen** | NSAID | Acidic |  | 0.97 | 5.94 |
| **Naproxen** | NSAID | Acidic |  | 3.18 | 4.53 |

**Table 3.2.1 (b): Characteristics of acidic, basic and neutral drugs from plasma matrix**

| Drug | Pharmacology | Classification | Structure | Log P | pKa |
|---|---|---|---|---|---|
| **Acetaminophen** | Analgesic | Neutral |  | 0.92 | 3.59 |
| **Amphetamine** | Stimulant | Basic |  | 1.71 | 9.8 |
| **p-Toluamide** | (used for pigments) | Acidic |  | 1.31 | 5.05 |
| **m-Toluidine** | Repellent | Basic |  | - | 9.8 |
| **Phenobarbital** | Sedative | Acidic |  | 1.47 | 7.2 |

## 3.3 Polycyclic Aromatic Hydrocarbons in food and environmental matrices

Polycyclic aromatic hydrocarbons, or polynuclear aromatic hydrocarbons (PAHs), are fused ring aromatic compounds classified by the number of carbon rings as well as their carcinogenicity. The two and three ring PAHs are less potent relative to several of the four, five and six ring PAHs. The four ring PAHs include chrysene and benzo[a]anthracene; the five ring PAHs, benzo[a]pyrene, benzo[b]fluoranthene, benzo [k] fluoranthene and dibenzo [a,h] anthracene, while the six ring PAH include indeno [1,2,3-cd] pyrene. Benzo [a] pyrene is the most potent carcinogen among the PAHs [115]. The US-EPA and EU lists sixteen of these PAHs (Fig. 3.3.1) as hazardous compounds [116]. Generally PAHs are lipophilic compounds that show a high affinity for organic matter, nonetheless some of them can dissolve quite well in water [117].

Most PAHs in the environment derive from incomplete combustion of carbon containing materials such as oil, wood, garbage or coal. A maximum amount of PAHs is formed when materials burn at temperatures in the range 500 – 700 $^{o}$C, as in wood and cigarettes [118]. Excluding smokers and occupationally exposed populations, most individuals are exposed to PAHs predominantly from dietary sources [119]. In the marine environment, PAHs are bioavailable to marine species via the food chain, as waterborne compounds and from contaminated sediments. Since PAHs are lipophilic compounds they easily cross lipid membranes and have the potential to bioaccumulate in aquatic organisms. Although for most people, fish and seafood represents only a small part of the total diet, the contribution of this food group to the daily intake of PAHs in some individuals may be comparatively important [120].

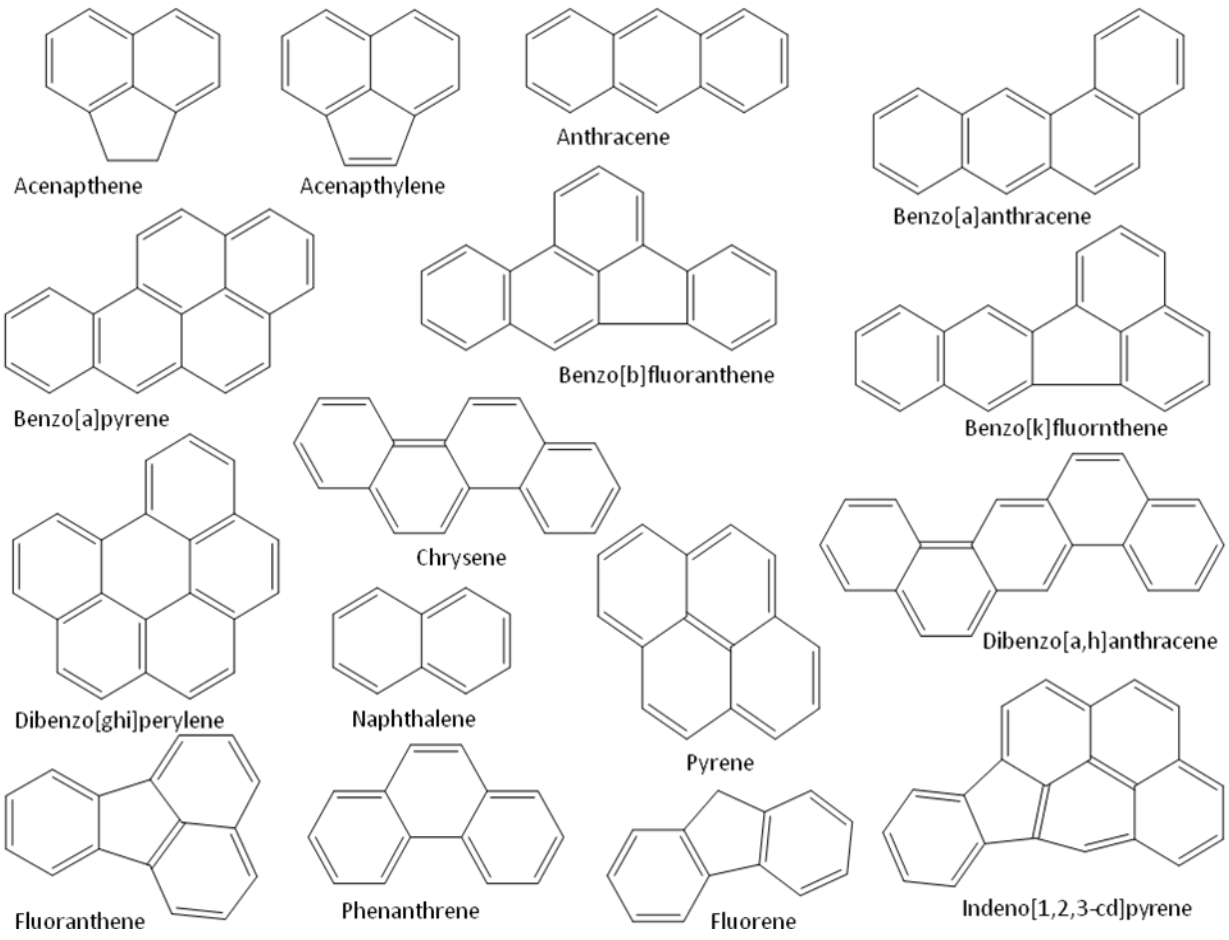



**Figure 3.3.1: Chemical structures for the 16 polycyclic aromatic hydrocarbons**

Several extraction methods (Soxhlet, LLE or SPE) have been investigated for sample preparation of soil and most of these involved an evaporation step. However, evaporation leads to the loss or low recoveries of the volatile PAHs such as naphthalene [120]. Microwave-assisted solvent extraction (MASE) and pressurized liquid extraction (PLE) are generally faster, less analyte- and matrix-dependent and provide cleaner extracts than conventional methods involving heat treatment. In a study to determine PAHs in soils and sediment a miniaturized PLE was employed in a static-dynamic extraction procedure. The procedure was optimised with regards to organic solvent choice, temperature and pressure. The performance of the set-up, which was combined at-line with gas chromatography–mass spectrometry (GC–MS), was evaluated. [121].

The extraction cell was built-in in a heatable 10 mm x 3.0 mm I.D. stainless steel holder (see Fig.3.3.2). It was sealed with a stainless-steel frit at its upper end (in the direction of solvent flow) to prevent clogging of the exit tubing and valve by soil/sediment particles. The reduced solvent volume, together with the use of large-volume injection (LVI), allowed the at-line coupling of the extraction and separation-plus-detection steps without the need for a concentration step prior to GC analysis.

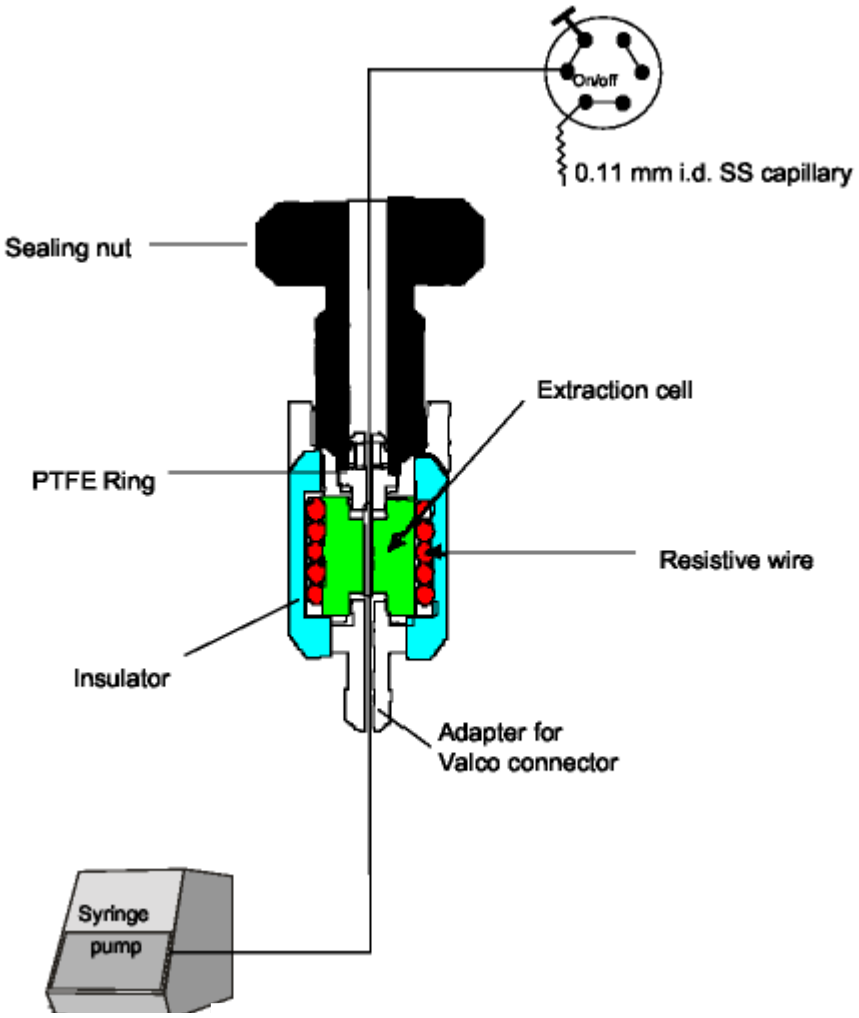



**Figure 3.3.2: PLE device for extraction of solid and semi-solid samples [121]**

PLE has also been employed for integrated exhaustive extraction of PAHs with fat removal from smoked fish [122]. The one-step procedure provided a more rapid and cost-efficient alternative with minimization of waste generation compared to the standard reference method that is based on a multi-step procedure. Furthermore, the integrated approach for extraction and cleanup was less prone to analytical errors (random and systematic) because of the fewer analytical steps.

One of the traditional sample preparation techniques for the extraction of PAHs for fish analysis is Soxhlet [123] and SFE [124]. Size-exclusion chromatography (SEC) clean-up technique has also been employed for PAH analysis in non-fatty solid food [125] and plant matrices [126]. Non-polar high molecular compounds such as PAHs [127, 128] and PCBs [129, 130] were extracted with subcritical heated water (PLEHW) at temperatures greater that 250 $^{\circ}$C.

The advantages and disadvantages of the extraction techniques frequently employed for PAH analyses are summarized in Table 3.3.1.

***Table 3.3.1: Advantages and disadvantages of extraction techniques [116]***

| Technique | Advantage | Disadvantage |
|---|---|---|
| Ultrasonic extraction | Short extraction time, simple | Limited extraction efficiency |
| Soxhlet | Simple | Labor intensive, time and solvent consumption, analysis of numerous samples is limited by the extraction step, limited extraction efficiency |
| Automated Soxhlet extraction | Time saving, less solvent consumption, economical, reproducible, easy operation | Instrument cost |
| Pressurized fluid extraction | Simple extraction protocol, less solvent and time consumption, short extraction time, easy operation | Instrument cost, safety |
| Pressurized hot water extraction | Nil use of hazardous solvents, environmentally friendly, high extraction efficiency | Instrument cost, safety |
| Microwave assisted extraction | Simple instrumentation, reduced solvent use, short extraction time | Instrument cost, subjected to interference of microwave energy absorbing materials, requires filtration after extraction. |
| Supercritical fluid extraction | Environmental friendly, high speed of analysis | High analytical cost |

A method based on the advantages of a QuEChERS procedure (quick, easy, cheap, effective, rugged and safe) has been reported for the determination of 16 PAHs from fish samples. For a selective measurement of the compounds, extracts were analysed by LC with fluorescence detection. The overall analytical procedure was validated by systematic recovery experiments at three levels and by using the standard reference material [131, 132].

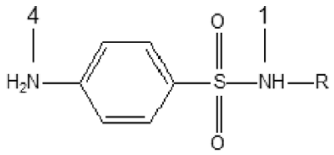
This thesis presents a method for the analysis of PAHs at trace levels in fish tissue and soil with HPLC-FLD. The HPLC methods are useful for PAH analysis since UV and fluorescence detection offer enhanced selectivity over other techniques such as GC with flame ionization detection [131].The method includes sample preparation with SampliQ QuEChERS AOAC Buffered Extraction kit (p/n 5982-5755) and SampliQ AOAC Fatty Dispersive SPE 15 mL kit (p/n 5982-5158).

## 3.4 Sulfonamides in food matrices

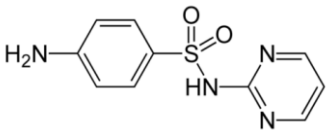
Sulfonamides (SAs) are a broad-spectrum of antimicrobial drugs used mainly in veterinary practice for therapeutic or prophylactic purposes in animals as well as in human beings [133]. In humans, sulfonamide antibiotics are commonly used for the therapy of infections. SAs maybe used in transplantation and for AIDS-related complications [134] alone or in combination with trimethoprim [135]. However, their use in human therapy has since become limited due to the advent of antibiotics [136]. They are a treatment of choice for disease control of coccidiosis in the poultry management [137]. SAs may also be used as additives in animal feed since prolonged ingestion of sulfonamides may have a growth-promoting effect [138].

Conversely, there is a health risk associated with consumption of animal products contaminated with sulfonamide residues. The residues usually result from the inappropriate administration or withdrawal period from these drugs. The presence of sulfonamide residues can trigger adverse side effects such as allergic reactions in hypersensitive individuals and are potential carcinogens in the long term. Furthermore, prolonged exposure to sulfonamide residues may give rise to an increase in drug-resistant bacteria [139]. In order to protect consumers from risks related to the drug residues, maximum residue limits (MRL) have been established by law in many countries. In Europe (EU Regulation 1999), Canada and USA (FDA Regulation 1991) the MRL for the total sulfonamides concentration in edible tissue is 100 μg/kg while it is 20 μg/kg in Japan [140, 141].

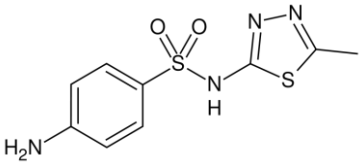
The basic chemical structure of sulfonamides is a common p – aminobenzoyl ring moiety with an aromatic amino group at the N1 – position. Fig. 3.4.1 shows the backbone and chemical structures of the sulfonamides described in this thesis.



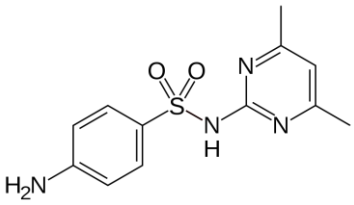
Sulfonamide backbone structure



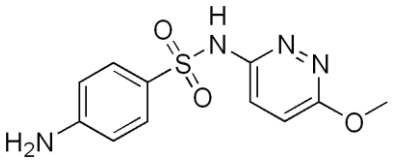
Sulfamerazine (pKa 6.98, logP 0.44)



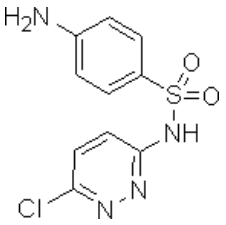
Sulfamethazine (pKa 7.45, logP 0.43)



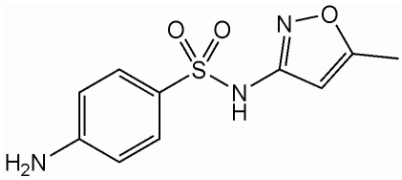
Sulfamethizole (pKa 5.51, logP 0.53)



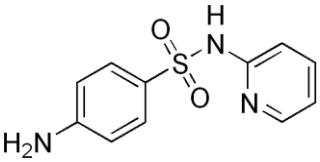
Sulfamethoxypyridazinep (Ka 7.19, logP 1.01)



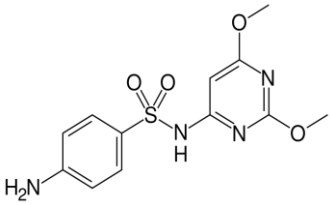
Sulfachloropyridazine (pKa 5.90, logP 1.36)



Sulfamethoxazole (pKa 5.81, logP 1.58)



Sulfapyridine (IS)



Sulfadimethoxine (pKa 6.21, logP 1.56)

**Figure 3.4.1: Chemical structures for the sulfonamides**

Centrifugal ultrafiltration [142], SPME [143], microdialysis system [144], LLE [145], on-line clean-up restricted access media columns [146] and SPE [147, 148] are some of the popular pre-concentration and matrix isolation techniques in analytical chemistry employed for the extraction of sulfonamides. Liquid-phase micro-extraction (LPME) has attracted increasing attention as a sample preparation technique. LPME is simple, low-cost, rapid, and requires only very small sample and solvent consumption [149]. In LPME, extraction normally takes place between small quantities of a water-immiscible solvent and an aqueous phase containing the analytes of interest. The volume of the acceptor phase is in the microliter or submicroliter range. Single-drop micro-extraction (SDME) has evolved from LPME, in which the extraction phase is in the form of a single drop suspended in the stirred aqueous solution. Several different operational techniques including static and dynamic-LPME [150, 151], hollow fibre membrane-LPME [152], solvent bar micro-extraction [153], continuous micro-extraction [154] and drop-to-drop solvent micro-extraction [155] have since been developed.

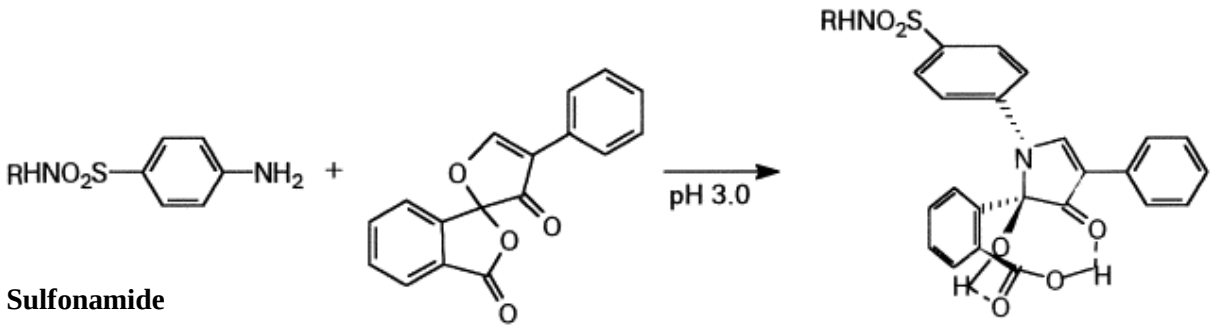
A salting-out assisted liquid extraction coupled with back-extraction by a water/acetonitrile/dichloromethane ternary component system combined with high-performance liquid chromatography with diode-array detection was developed for the extraction and determination of sulfonamides in solid tissue samples [156]. The procedure entailed homogenization of the swine muscle, centrifugation and back-extraction.

A stir bar sorptive extraction coupled to high performance liquid chromatography with diode array detection has also been employed for the quantitative monitoring of sulfonamide antibacterial residues in milk [157]. The analytes were concentrated by SBSE based on poly (vinylimidazole–divinylbenzene) monolithic material as coating. The extraction procedure was very simple; milk was diluted with water then directly extracted without elimination of fats and protein in samples. To achieve optimum extraction for SAs, several parameters, including extraction, desorption time, desorption solvent, ionic strength and pH value of sample matrix were investigated.

There are several analytical methods that include HPLC [158, 159], GC [160] and CE [161] for the determination of sulfonamides. In addition, mass spectrometry (MS), ultraviolet (UV) and fluorescence (FL) detectors have been used for SAs [162]. Higher sensitivity can be obtained on MS but higher cost will be paid in instrument and analysis procedure. Therefore, HPLC with UV or FL detection is most frequently applied [163, 164, 165, 166].

Fluorescence detector is a good alternative to MS, mainly due to its inherent sensitivity. However, the target compounds need prior derivatization with an appropriate reagent. In this way, post-column derivatization with fluorescamine has been mostly applied for the HPLC determination of sulfonamides [167].

Fluorescamine is a fluorogenic reagent specific for primary aliphatic and aromatic amines. It produces fluorophors of a high fluorescence yield and potential selectivity having an essentially similar excitation–emission spectral characteristic ($\lambda_{ex}$=395–410 nm and $\lambda_{em}$=490–510 nm). Fluorescamine and its hydrolysis products are non-fluorescent [168]. The concentration of fluorescamine and reaction time for the derivatization of SAs are factors to consider in order for results to be reproducible [169,170, 171]. Furthermore, at 2.5–3.5 pH range hydrogen bonding exists and the derivatives acquire fluorescence properties (see Fig. 3.4.3).




**Sulfonamide**

**Sulfonamide derivative**

**Figure 3.4.3: A depiction of a chemical reaction for the derivatization of sulfonamides [168].**

A method for the determination of sulfonamide drugs in chicken muscle with HPLC-FLD after a pre-column derivatization with fluorescamine, is presented in this thesis. The method includes sample preparation with SampliQ QuEChERS AOAC Buffered Extraction kit (p/n 5982-5755) and SampliQ AOAC Fatty Dispersive SPE 15 ml kit (p/n 5982-5156).

## 3.5 Acrylamide in food matrices

Acrylamide (AA) is an organic compound that is odorless, soluble in water, ethanol and ether. It has the composition of an amide, with the chemical formula $C_3H_5NO$ (Fig. 3.5.1). Acrylamide is used to manufacture plastic materials, paper, dyes as well as been used in the textile industry. It is also used for gel electrophoresis and has been used as a monomer in the synthesis of polyacrylamide. Polyacrylamide is used in the purification of water and in the formulation of grouting agents. Acrylamide is known as a component in tobacco smoke [172].

Acrylamide occurs naturally as a by-product of the cooking process and its presence in food was first confirmed by Swedish researchers in 2002 [173]. The Swedish findings about the high levels of acrylamide in heat treated foods were confirmed by the UK Food Standards Agency. The US Environmental Protection Agency (USEPA) found the limit for acrylamide in drinking water to be extremely low (0.5 µg/kg) [174]. Carbohydrate-rich foods such as French fries processed at high temperatures and under low moist conditions were of a great concern as high concentrations of acrylamides were produced [175]. Acrylamide, at high concentrations, has adverse effects. It is a human neurotoxin and has also been classified as a probable carcinogen and genotoxicant [176]. Acrylamide vapors irritate the eyes and the skin and also cause paralysis of the cerebrospinal system [177].

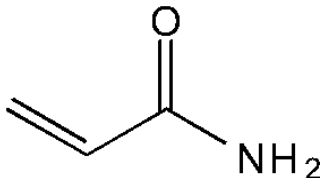
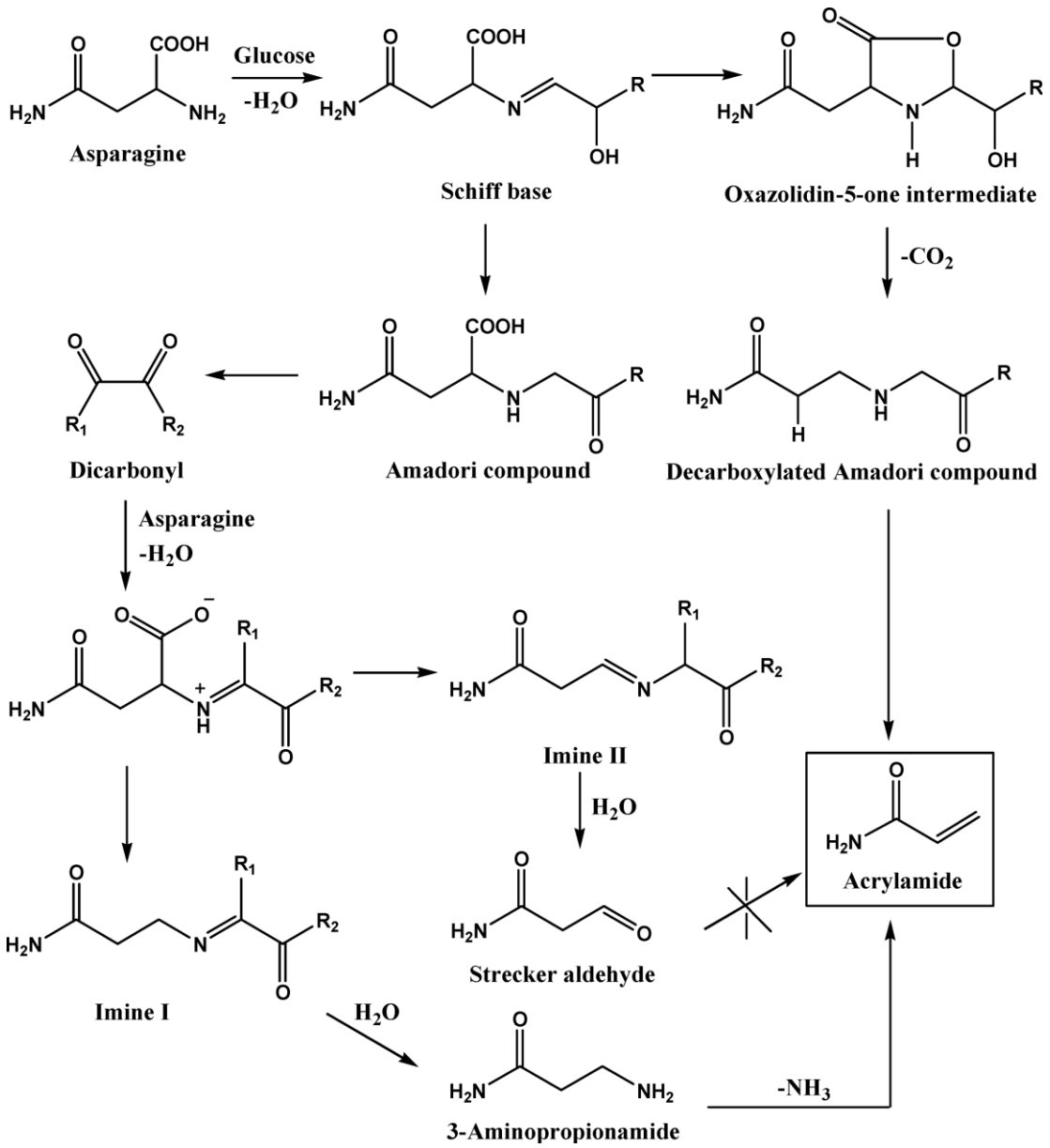



**Figure 3.5.1: Chemical structure of acrylamide( pKa 5.5, logP 0.67)**

The main approach for the formation of acrylamide in foods is through the Maillard reaction [178]. In summary, the mechanism involves the formation of a Schiff base. This is followed by the decarboxylation and elimination of either ammonia or a substituted imine to yield acrylamide. Mass spectral studies have shown that the three carbon atoms and the nitrogen atom of acrylamide are all derived from asparagines [179]. The first critical step is the amino-carbonyl reaction between asparagine and a carbonyl substance, preferably $\alpha$-hydroxycarbonyls (e.g. reducing sugars). Finally a Schiff base, a key intermediate, is formed after dehydration under elevated temperatures (see scheme 3.5.1).

Several methods have been developed to determine the acrylamide monomer, especially in water, biological fluids and food [180]. The majority of these methods were based on liquid (LC) or gas chromatographic (GC) techniques [181 - 184]. However, these methods lacked the selectivity to confirm the presence of a small molecule such as acrylamide in complex matrices. A more selective method based on size-exclusion chromatography coupled with electrospray mass spectrometry was developed for the determination of acrylamide in fried foods [185].




**Scheme 3.5.1: Formation and reduction of acrylamide [178]**

Liquid chromatography coupled to diode array detection (LC–DAD) has also been used accurately and precisely, as an alternative to tandem LC–MS methods for the determination of acrylamide in potato-based foods at low levels [186]. The method entailed extraction of acrylamide with methanol, purification with Carrez I and II solutions, an evaporation step and clean-up with an Oasis HLB solid-phase extraction cartridge. The chromatographic separations were performed on a hydrophilic and a hydrophobic interaction columns having good retention of acrylamide (*k* 3.67 and 2.54, respectively). Clean-up steps often employ SPE procedures that are compatible with LC-MS/MS and GC-MS without any solvent exchange (evaporation) and/or derivatization prior to the determinative step [187].

A stepwise study was carried out on the common factors that influence the extraction of acrylamide from different food matrices [188]. The investigated extraction factors included sample particle size (fine or coarse), defatting, extraction solvent (water or water/methanol), homogenization, extraction temperature and extraction time. An optimised method comprised the use of fine particles (<1000 μm), water as the extraction solvent and shaking of the sample. This extraction method was suitable for all tested matrices (coffee, crisp-bread, mashed potatoes, milk chocolate and potato crisps).

The analytical results (from LC-MS/MS analysis after SPE clean-up) correlated well with those obtained by the original, more labor-intensive and extraction procedure. There was excellent agreement with the assigned AA levels of several proficiency test samples analysed for evaluation. Defatting or the additional homogenisation did not have any observable effect on the AA yield. In general, the study revealed that incomplete extraction is the most likely cause of erroneous results. Incomplete extraction may occur when the food is not sufficiently macerated and when water/methanol is used as the extraction solvent. In addition, incomplete extraction may occur when using a short extraction time or when the extraction temperature is low. Formation of AA during the extraction procedure is another possible error source.

A sample preparation protocol that employed elements from the QuEChERS method, such as dispersive-SPE clean-up, was evaluated for the extraction of acrylamide from various food matrices [189]. The optimized procedure included solubilising the samples with hexane, addition of water and acetonitrile for the extraction of acrylamide. A salt mixture of anhydrous $MgSO_4$ and NaCl was added to induce solvent-phase separation. Fig. 3.5.2 depicts the arrangement of solvents after centrifugation. For clean-up the acetonitrile extract was added to a mixture of PSA and $MgSO_4$ and the aliquot subsequently analysed with LC-MS/MS or GC-MS.

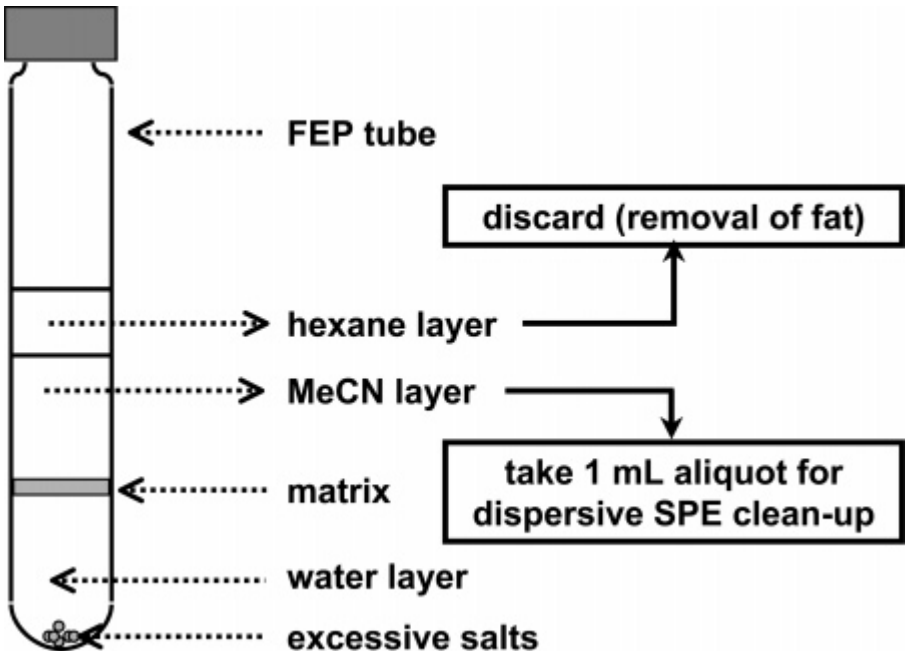



**Figure 3.5.2: Schematic picture of the solvent layer arrangement in a FEP tube after the centrifugation of a food extract [189].**

The highest overall partition of acrylamide (>70%) was achieved after adding 4 g of $MgSO_4$ and 0.5 g of NaCl (Fig. 3.5.3), which was consistent to the previous findings. A combination of $MgSO_4$ and NaCl induced a distinct phase separation between water and MeCN. In addition, the salt combination stimulated most pesticides to partition into the upper MeCN layer (salting out mechanism) [190]. It was reported that in real sample analysis, salts and other polar food components can slightly influence acrylamide partitioning. However, the use of an isotopic labeled internal standard (*d*3-acrylamide) provided an effective compensation for potential variability in acrylamide partitioning efficiency.

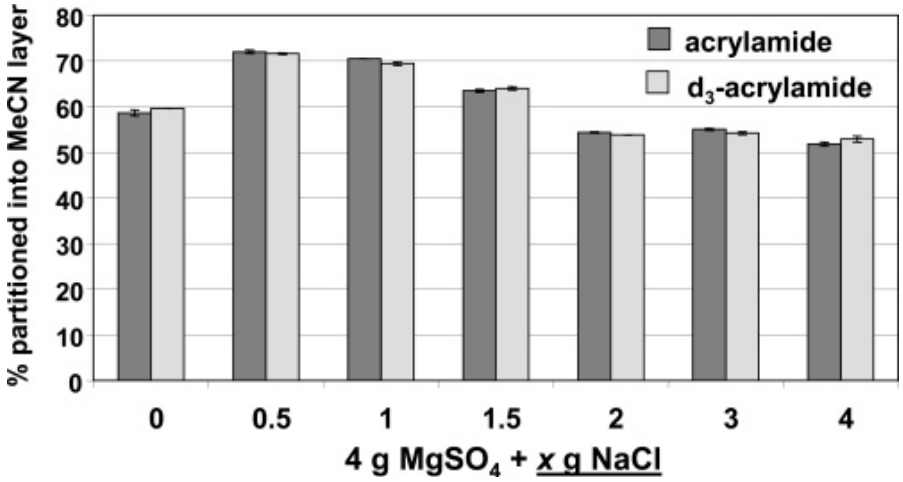



**Figure 3.5.3: Partition of acrylamide and d3-acrylamide (in %) into the MeCN layer in the experiments involving addition of 4 g of MgSO4 and 0-4 g of NaCl to 50 ng/ml composite solutions of acrylamide and d3-acrylamide in water-MeCN [189]**

This thesis presents a method for the analysis of acrylamide in cooking oil with HPLC-DAD. The method includes sample preparation with SampliQ QuEChERS Extraction kit for acrylamides (p/n 5982-5850) and SampliQ EN for fruits and vegetables with fats and waxes Dispersive SPE kit (p/n 5982-5156).

# CHAPTER 4

# Experimental

## 4.1 Overview

Commercial polymeric mixed-mode sorbents were employed for fractionating acidic, basic and neutral drugs; secobarbital, nortriptyline, ketoprofen and naproxen from urine and amphetamine, acetaminophen, p-toluamide, m-toluidine and phenobarbital from plasma while the QuEChERS kits were employed for the analysis of PAHs (naphthalene , acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, 1,2-benza[a]anthracene, chrysene, benzo[e]pyrene, benzo[e]acenaphthylene, benzo[k]fluoranthene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene) in fish and soil samples; for the determination of sulfonamide residues (sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxypyridazine, sulfachloropyridazine, sulfamethoxazole and sulfadimethoxine) in chicken and for the determination of acrylamide in cooking oil.

## 4.2 Chemicals, Reagents and Standards

### Chemicals and Reagents

Ketoprofen, secobarbital, nortriptyline, naproxen, acetaminophen, phenobarbital, p-toluamide, amphetamine, m-toluidine, ranitidine (IS), the 16 polycyclic aromatic hydrocarbons, 10 sulfonamides, acrylamide and methacrylamide were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All reagents were analytical or HPLC grade. Acetonitrile, acetone, n-hexane and glacial acetic acid (HAc) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphoric acid, formic acid and potassium hydroxide were from Merck Chemicals (Gauteng, South Africa) while the HPLC grade methanol (MeOH) was from Merck KGaA (Darmstadt, Germany). Potassium hydrogen phosphate, potassium dihydrogen phosphate and sodium acetate were purchased from Saarchem Analytical (Krugersdorp, South Africa). Fluorescamine with a purity of 98% was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Standard Solutions

The mobile phases were prepared with ultrapure water (18.2 MΩcm) from a MilliQ system by Millipore (Milford, Mass, USA) and filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size). The stock solutions (1 mg/ml) were prepared in either acetonitrile or methanol and kept at 4 °C while the working solutions were prepared daily by diluting the stock solutions, to appropriate concentrations, also in acetonitrile or methanol for all drugs with the exception of sulfonamides.

For the sulfonamides, a stock solution of 0.05 M sodium acetate was prepared by dissolving 4.1 g NaOAc in 1 L ultrapure water and filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size). The pH was adjusted using HAc. Fluorescamine reagent (0.02%) was prepared by dissolving 20 mg Fluram in 10 ml acetone. The solution was stored at 4 °C. 1% HAc in MeCN was prepared by adding 10 ml HAc in 1 L MeCN while working solutions were prepared daily by serial dilution in 0.05 M NaOAc (pH 3.5). The solution vials were wrapped-up with an aluminum foil as some of the sulfonamide drugs are light sensitive.

The urine sample was from a donor who is not using or has not used the drugs in the study and the plasma sample was from SANBS (Port Elizabeth, South Africa). Food samples were purchased from the local supermarkets while the soil sample was obtained from a botanical garden.

## 4.3 Equipment and Material

The Agilent 1200 Series HPLC was equipped with a binary pump and different detection modes: a diode array detector (DAD) set at $\lambda$ = 222 nm for drugs in urine; at $\lambda$ = 210 nm for drugs in plasma; and at $\lambda$ = 210 nm for acrylamide in cooking oil. Separation of the compounds in urine and plasma samples was achieved on an Agilent ZORBAX Eclipse Plus $C_{18}$ column (4.6 mm x 75 mm, 3.5 µm) while that of acrylamide and methacrylamide (IS) was achieved on an Agilent ZORBAX HILIC Plus column (4.6 mm x 50 mm, 3.5 µm). A fluorescence detector (FLD) set at varying excitation and emission wavelengths was employed for PAHs in the fish and soil samples while separation of the PAHs was achieved on an Agilent ZORBAX Eclipse PAH $C_{18}$ column (4.6 mm x 50 mm, 1.8 µm). For sulfonamides, FLD was set at $\lambda_{ex}$ = 405 nm and $\lambda_{em}$ = 495 nm and separation of SAs was achieved on an Agilent ZORBAX Eclipse Plus $C_{18}$ column (4.6 mm x 75 mm, 3.5 µm). The esquire™ series mass spectrometer was operated in positive electrospray ionisation mode (ESI (+)) for identification of SAs. The data was processed by Agilent Chemstation for LC/MS 2D system software.

A Jenway 3510 pH meter (London, UK) was employed to monitor the pH of solutions and a Kenwood grinder (Grahamstown, South Africa) for homogenising the food sample.

The SPE materials were supplied by Agilent Technologies Inc. (CA, USA);

- Agilent SampliQ SAX, 1 ml/30 mg containing a water-wettable polymeric anion exchanger with 25 - 35 μm average particle size. SampliQ SAX resin is a tertiary amine modified divinyl benzene polymer that exhibits a dual retention mechanism, strong anion exchange (for both acidic and neutral analytes over a range of hydrophobicity, log P) and a reversed phase behavior.

- Agilent SampliQ SCX, 1 ml/30 mg and a polymeric strong cation exchanger with 25 – 35 μm average particle sizes. The polymeric backbone, sulfonic acid modified divinyl benzene is also water-wettable with strong cation exchange and hydrophobic mechanism.

- Agilent SampliQ Buffered QuEChERS AOAC Extraction kit, p/n 5982-5755 and SampliQ QuEChERS AOAC Dispersive SPE kit, p/n 5982-5058.

- Agilent SampliQ QuEChERS Extraction kit for acrylamides, p/n 5982-5850 and SampliQ QuEChERS EN Dispersive SPE kit, p/n 5982-5165.

## 4.4 Chromatographic conditions

**Table 4.4.1: HPLC conditions for drugs in urine**

| | |
|---|---|
| **Column** | Agilent ZORBAX Eclipse Plus C18 4.6 x 75 mm, 3.5 µm |
| **Flow rate** | 1.5 ml/min |
| **Column temperature** | 30 ˚C |
| **Injection volume** | 5 µl |
| **Mobile phase** | Isocratic elution<br>A: 55% $CH_3OH$<br>B: 45% 25 mM $KH_2PO_4$ pH 2.7 |
| **Run time** | 8 min |

**Table 4.4.2: HPLC conditions for drugs in plasma**

| | |
|---|---|
| **Column** | Agilent ZORBAX Eclipse Plus C18 4.6 x 75 mm, 3.5 µm |
| **Flow rate** | 1.5 ml/min |
| **Column temperature** | 35°C |
| **Injection volume** | 5 µl |
| **Mobile phase** | Isocratic elution:<br>A: 30% $CH_3OH$<br>B: 70% 25 mM $KH_2PO_4$ / $K_2HPO_4$ pH 7 |
| **Run time** | 6 min |

**Table 4.4.3: HPLC conditions for PAHs in fish and soil**

| | |
|---|---|
| **Column** | Agilent ZORBAX Eclipse PAH C18 4.6 x 50 mm, 1.8 μm |
| **Flow rate** | 0.8 ml/min |
| **Column temperature** | 18 °C |
| **Injection volume** | 5 μl |
| **Mobile phase** | A = Deionized $H_2O$ B = $CH_3CN$ |
| **Gradient** | |

| T (min) | % B |
|---|---|
| 0 | 60 |
| 1.5 | 60 |
| 7 | 90 |
| 13 | 100 |

**Detection** UV at 230 nm (Acy) and varying fluorescence excitation and emission Wavelengths:

| Time (min) | Ex / Em Wavelengths (nm) | PAH detected |
|---|---|---|
| 0 – 5 | 260 / 352 | Nap, Ace, Flu, Phe, Chr |
| 0 – 14 | 260 / 420 | Ant, Pyr, BeP, DahA, BghiP |
| 0 – 14 | 260 / 460 | Fln, 1,2-BaA,BeA, BkF, InP |

**Table 4.4.4 (a): HPLC conditions for sulfonamide residues in chicken muscle**

| | | |
|---|---|---|
| **Column** | Agilent ZORBAX Eclipse Plus C18 4.6 x 75 mm, 3.5 μm | |
| **Flow rate** | 1 ml/min | |
| **Column temperature** | 25 °C | |
| **Injection volume** | 5 μl | |
| **Mobile phase** | A = 0.05 M Sodium Acetate pH 4.5 | B = $CH_3CN$ |
| **Gradient** | | |
| | T (min) | % B |
| | 0 | 35 |
| | 35 | 41 |
| | 50 | 55 |
| **Detection** | Ex = 405 nm | Em = 495 nm |

**Table 4.4.4 (b): Conditions for separation and analysis**

| **HPLC conditions** | |
|---|---|
| **Column** | Agilent ZORBAX SB- C18 2.1 x 30 mm, 3.5 μm |
| **Flow rate** | 0.3 ml/min |
| **Column temperature** | 40 °C |
| **Injection volume** | 5 μl |
| **Mobile phase** | A = 10 mM formic acid B = $CH_3OH$ |
| **Gradient** | |
| | T (min) % B |
| | 0 – 3 5 |
| | 10 15 |
| | 10.10 5 |
| **Detection** | $\lambda_=$ 270 nm |
| **MS conditions** | |
| **Polarity** | positive |
| **Gas Temperature** | 350 °C |
| **Gas Flow** | 9 L/ml |
| **Nebulizer** | 40 psi |
| **Capillary** | 4000 V |

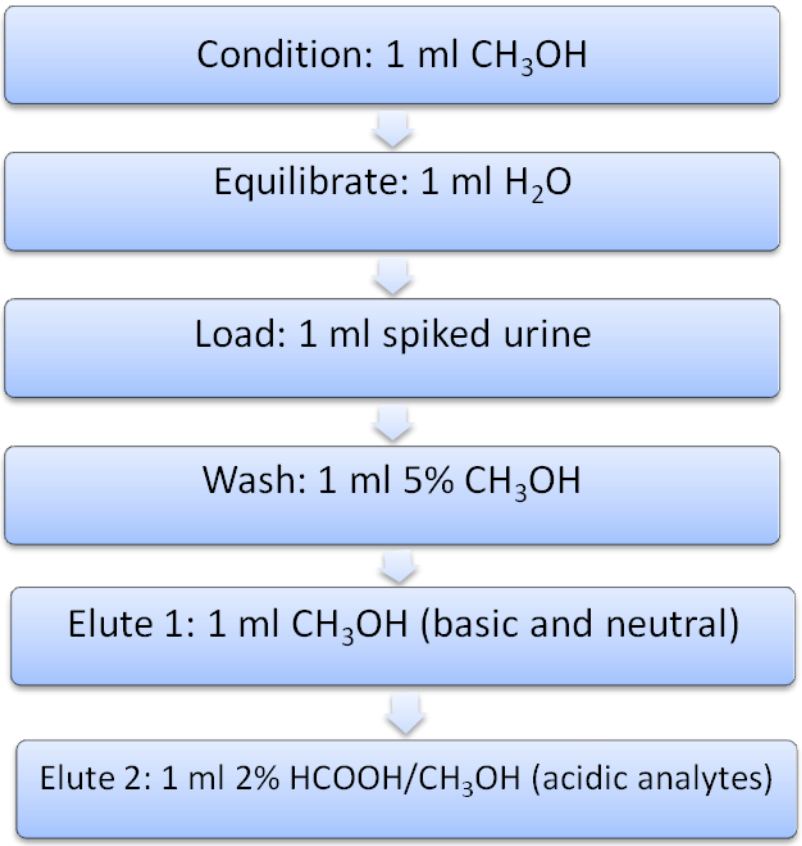
**Table 4.4.5: HPLC conditions for acrylamide in cooking oil**

| | |
|---|---|
| **Column** | Agilent ZORBAX HILIC Plus 4.6 x 50 mm, 3.5 μm |
| **Flow rate** | 0.2 ml/min |
| **Column temperature** | 30 °C |
| **Injection volume** | 5 μl |
| **Mobile phase** | Isocratic elution: A = 3% 5 mM Acetic acid B = 97% $CH_3CN$ |
| **Run time** | 10 min |
| **Post time** | 3 min |
| **Detection** | DAD @ 210 nm |

## 4.5 Sample preparation

### *4.5.1 Sample pretreatment for urine analysis*

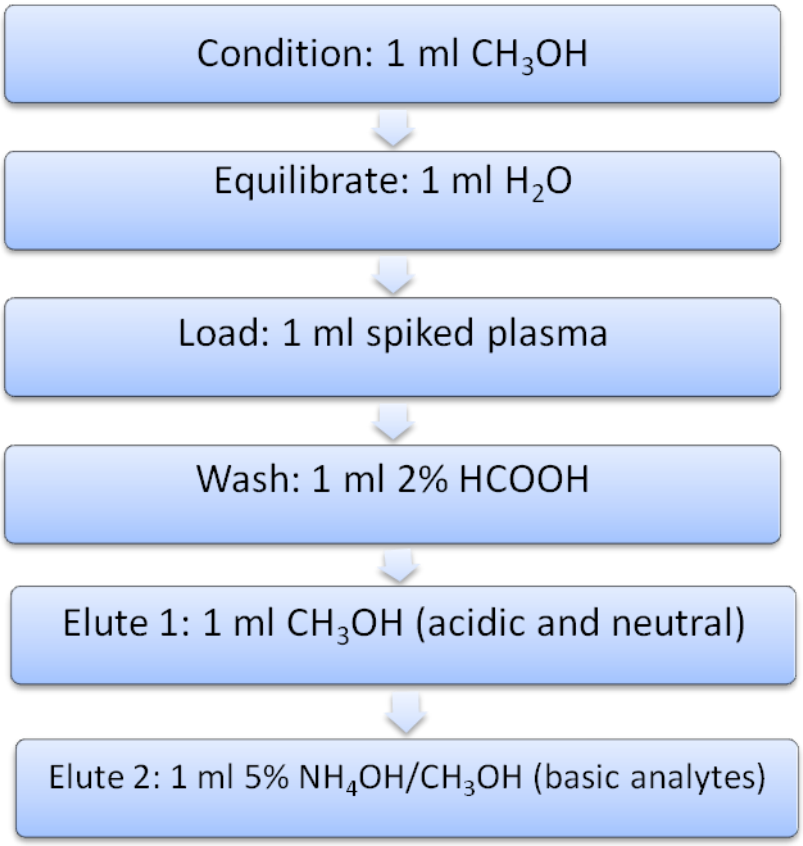
The urine (5 ml) was hydrolyzed with 1 M KOH at 60 ºC for 15 min and diluted with 10 mM $CH_3COONa$ (1:1 v/v) and the pH adjusted to 2 with phosphoric acid. The urine sample, spiked with drugs, was loaded onto the SampliQ SAX cartridges, as shown in Scheme 4.5.1.




**Scheme 4.5.1: SPE procedure (urine)**

### *4.5.2 Sample pretreatment for plasma analysis*

The plasma sample (1 ml) was hydrolysed with 1% formic acid (3 ml) for 30 min. An internal standard, 50 µl ranitidine was then added. The sample, spiked with drugs, was then loaded onto the SampliQ SCX cartridges, as described in Scheme 4.5.2 below:




**Scheme 4.5.2: SPE procedure (plasma)**

### *4.5.3 Sample preparation for PAHs in fish analysis*

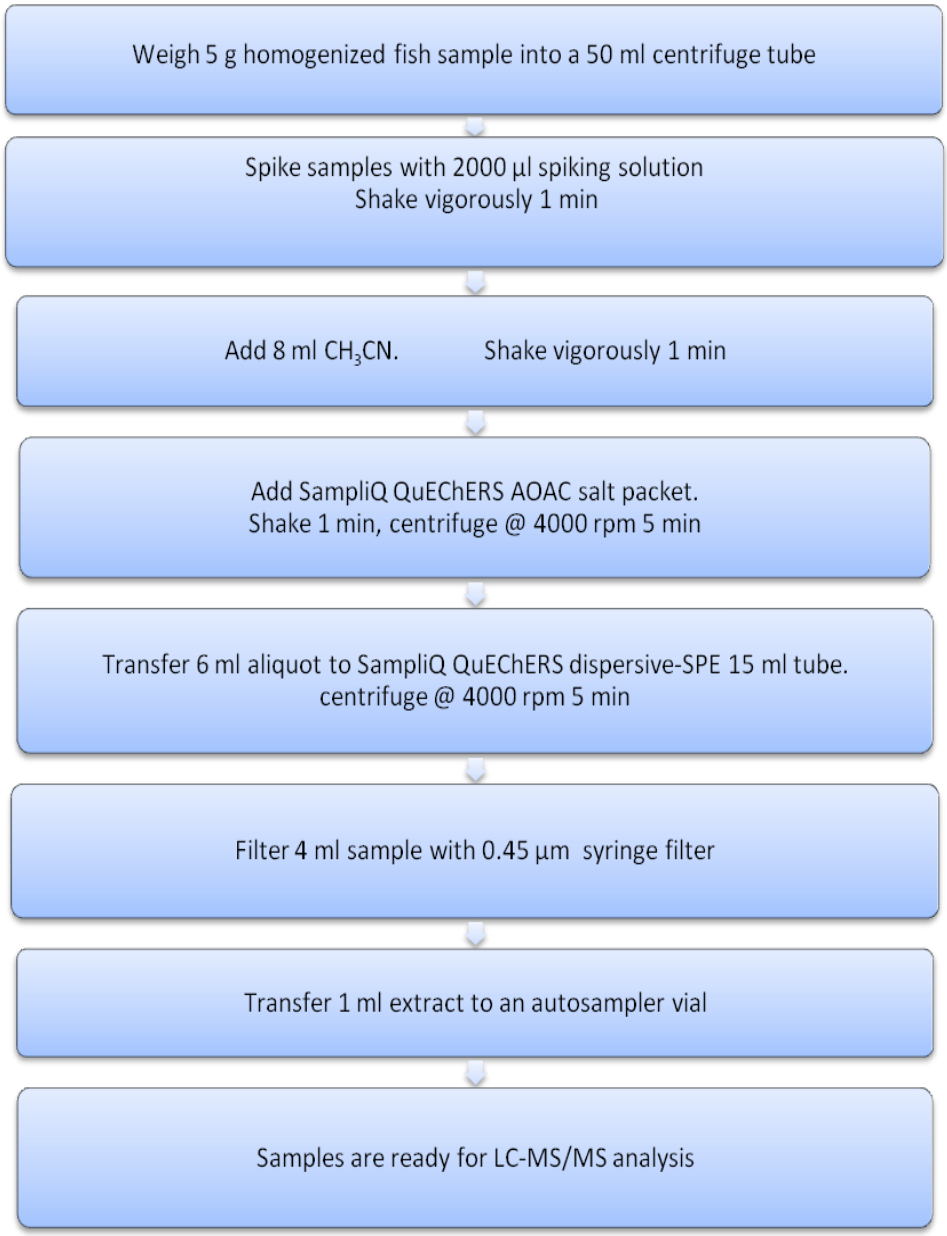
The fish fillets were minced and deep frozen until analysis.

***Extraction***

5 g fish sample homogenate was placed into a 50 ml centrifuge tube from the SampliQ QuEChERS AOAC Extraction kit and the tube centrifuged for 20 s. Samples were then spiked with appropriate spiking solutions to yield working solutions for recoveries and reproducibility studies. A 2000 µl spiking solution was added to the samples except the blank. After shaking vigorously for 1 min, 8 ml $CH_3CN$ was added, then an Agilent SampliQ QuEChERS AOAC extraction salt packet (p/n 5082-5755) was added. The packet contained 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous NaOAc. The sample tubes were hand shaken vigorously for 1 min and then further centrifuged at 4000 rpm for 5 min.

***Dispersive SPE cleanup***

6 ml of the upper $CH_3CN$ layer was transferred into a SampliQ QuEChERS AOAC Dispersive SPE 15 ml tube. This SPE tube contained 400 mg of PSA and 1200 mg of anhydrous $MgSO_4$. The tubes were then centrifuged at 4000 rpm for 5 min. 4 ml of the extract was filtered through a 0.45 µm PVDF syringe filter, then 1000 µl extract was placed in an autosampler vial foe an HPLC-FLD analysis. The QuEChERS protocol for PAHs in fish is shown in Scheme 4.5.3:




**Scheme 4.5.3: Flow chart of QuEChERS AOAC sample preparation procedure (fish)**

### *4.5.4 Sample preparation for PAHs in soil*

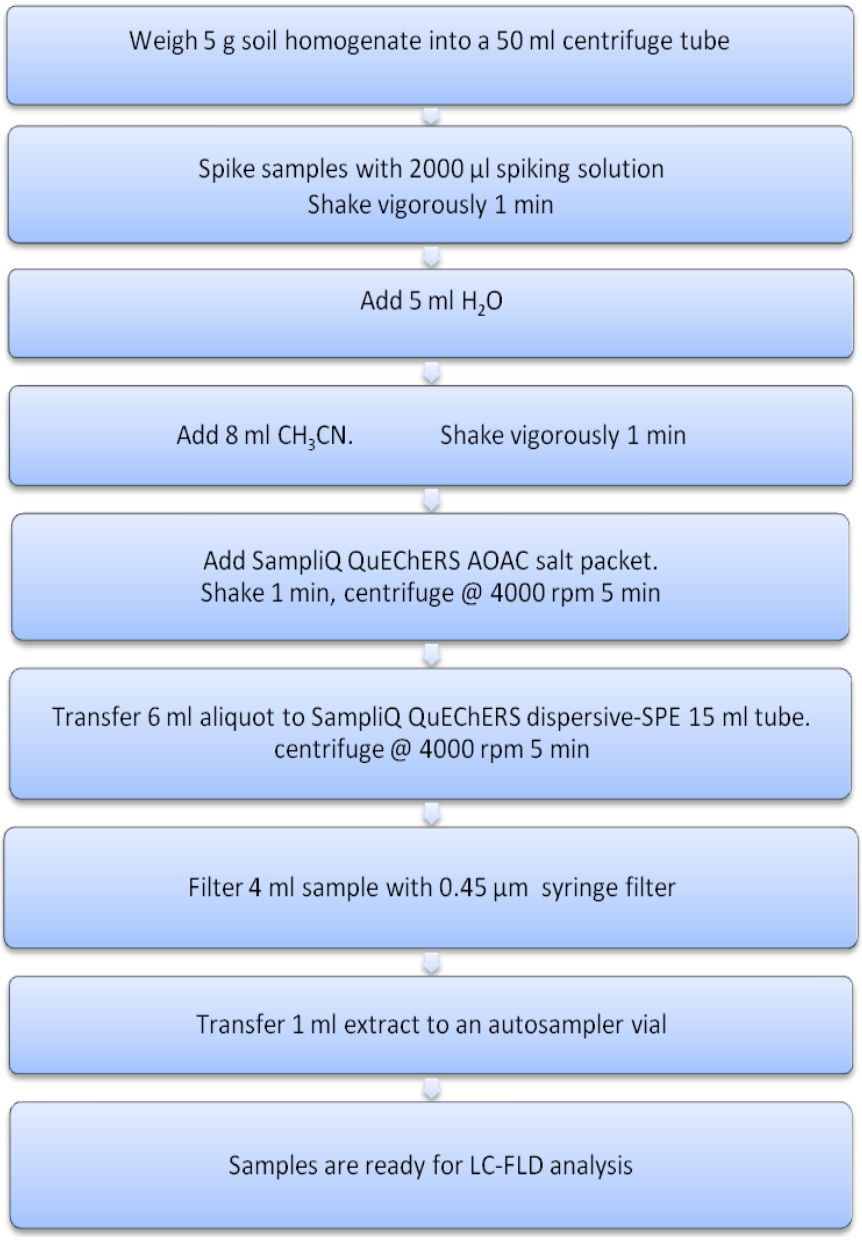
The soil sample was air dried at ambient temperature then sieved to obtain a homogeneous sample.

***Extraction***

5 g soil sample homogenate was placed into a 50 ml centrifuge tube from the SampliQ QuEChERS AOAC Extraction kit. Samples were spiked with appropriate spiking solutions to yield appropriate working solutions for recoveries and reproducibility studies. 2000 µl spiking solution was added to the samples except the blank. 5 ml water was then added to the tube. After shaking vigorously for 1 min, 8 ml $CH_3CN$ was added, then an Agilent SampliQ QuEChERS AOAC extraction salt packet (p/n 5082-5755) was added. The packet contained 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous NaOAc. The sample tubes were hand shaken vigorously for 1 min then further centrifuged at 4000 rpm for 5 min.

***Dispersive SPE cleanup***

6 ml of the upper $CH_3CN$ layer was transferred into a SampliQ QuEChERS AOAC Dispersive SPE 15 ml tube. This SPE tube contained 400 mg of PSA and 1200 mg of anhydrous $MgSO_4$. The tubes were then centrifuged at 4000 rpm for 5 min. 4 ml of the extract was filtered through a 0.45 µm PVDF syringe filter, then 1000 µl extract was placed in an autosampler vial for an HPLC-FLD analysis. Flow chart for the QuEChERS AOAC sample preparation procedure for PAHs in soil is shown in scheme 4.5.4:




**Scheme 4.5.4: Flow chart of QuEChERS AOAC sample preparation procedure (soil)**

### *4.5.5 Sample preparation for Sulfonamide residues in chicken muscle*

The chicken muscle was minced and deep frozen until analysis.

***Extraction***

2 g chicken muscle homogenate was placed into a 50 ml centrifuge tube from the SampliQ QuEChERS AOAC Extraction kit and the tube centrifuged for 20 s. Samples were then spiked with appropriate spiking solutions to yield 50, 100 150 ng/g sample concentrations for recoveries and reproducibility studies. A 100 µl IS spiking solution was added to all the samples except the blank. After shaking vigorously for 1 min, 8 ml Milli-Q water was added followed by shaking the mixture for 30 s. 10 ml 1% HAc in $CH_3CN$ was then added, after which an Agilent SampliQ QuEChERS AOAC Extraction salt packet (p/n 5082-5755) was added. The packet contained 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous NaOAc. The sample tubes were hand shaken vigorously for 1 min then futher centrifuged at 4000 rpm for 5 min.

***Dispersive SPE cleanup (HPLC-FLD)***

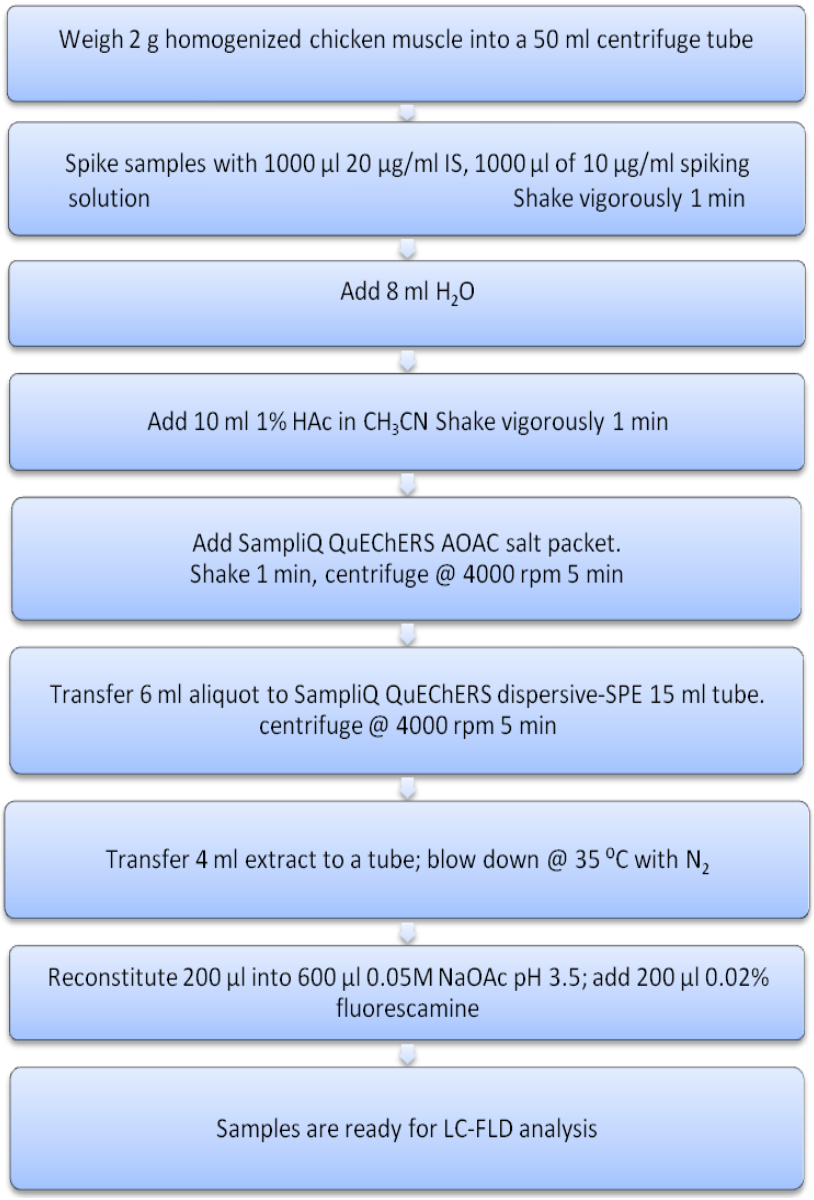
6 ml of the upper $CH_3CN$ layer was transferred into a SampliQ QuEChERS AOAC Dispersive SPE 15 ml tube. This SPE tube contained 400 mg of PSA and 1200 mg of anhydrous $MgSO_4$. The tubes were then centrifuged at 4000 rpm for 5 min. 4 ml of the extract was transferred to a test tube and dried with $N_2$ gas at 35 ºC. Samples (200 µl) were reconstituted into 600 µl of 0.05 M NaOAc (pH 3.5).

### *Derivatization*

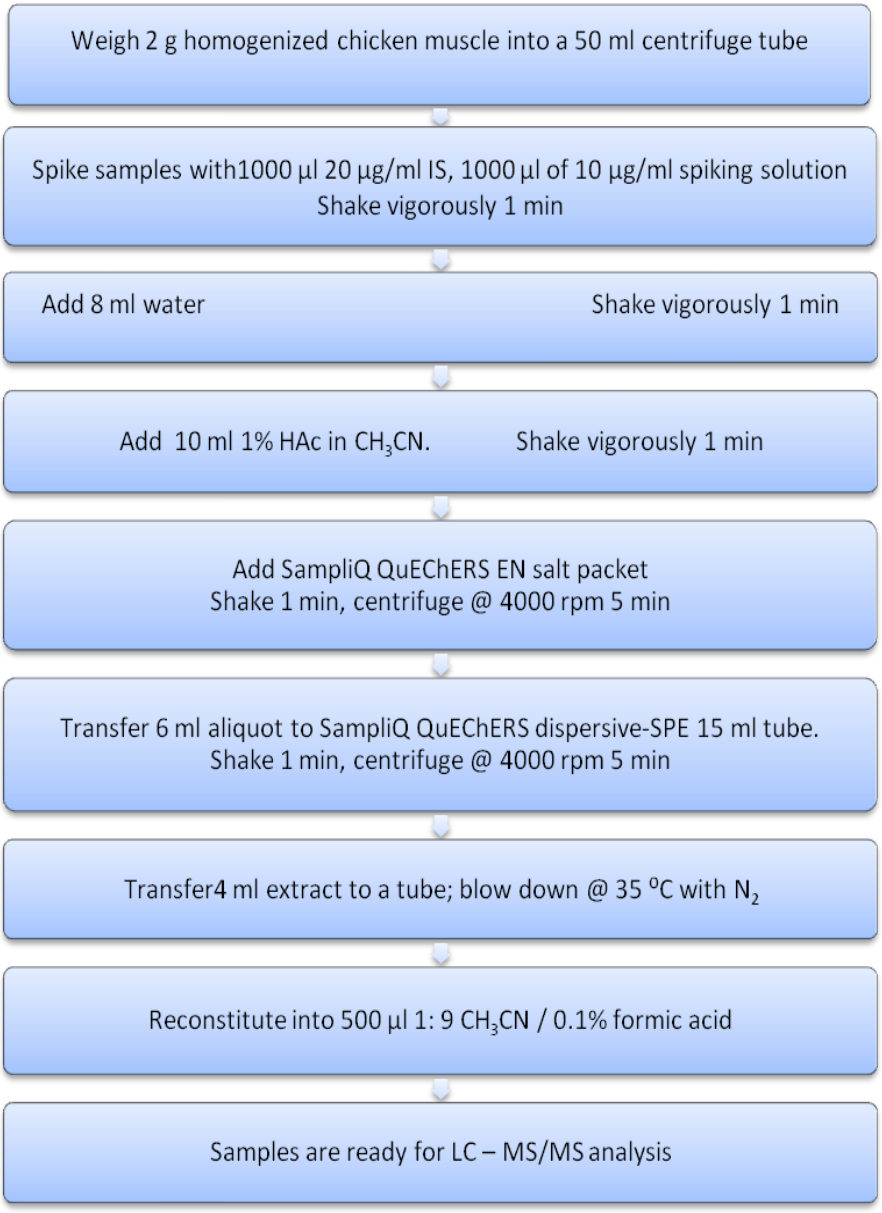
Aliquots of 200 µl working standard mixtures of sulfonamides, dissolved in 600 ml 0.05 M acetate buffer (pH 3.4) were filtered through a 0.45 µm PVDF syringe filter then transferred to reaction vials and 200 µl 0.02% w/v fluorescamine solution in acetone added. The mixtures were shaken for 1 min and the reaction left to proceed for 60 min at ambient temperature. Aliquots of 1000 µl of the derivatized solutions were directly injected into the chromatograph. Scheme 4.5.5 (a) shows the flow chart for the QuEChERS AOAC sample preparation procedure.

### *Dispersive SPE cleanup (LC-MS/MS)*

6 ml of the upper $CH_3CN$ layer was transferred into a SampliQ QuEChERS EN Dispersive SPE 15 ml tube. This SPE tube contained 150 mg PSA, 150 mg $C_{18}EC$ and 900 mg anhydrous $MgSO_4$. The tubes were then centrifuged at 4000 rpm for 5 min. 4 ml of the extract was transferred to a test tube and dried with $N_2$ gas at 35 ºC. Samples were reconstituted into 500 µl of 1: 9 $CH_3CN$ / 0.1% formic acid (Scheme 4.5.5b).




**Scheme 4.5.5 (a): Flow chart of QuEChERS AOAC procedure (LC-FLD analysis)**




**Scheme 4.5.5 (b): Flow chart of QuEChERS AOAC procedure (LC-MS/MS analysis)**

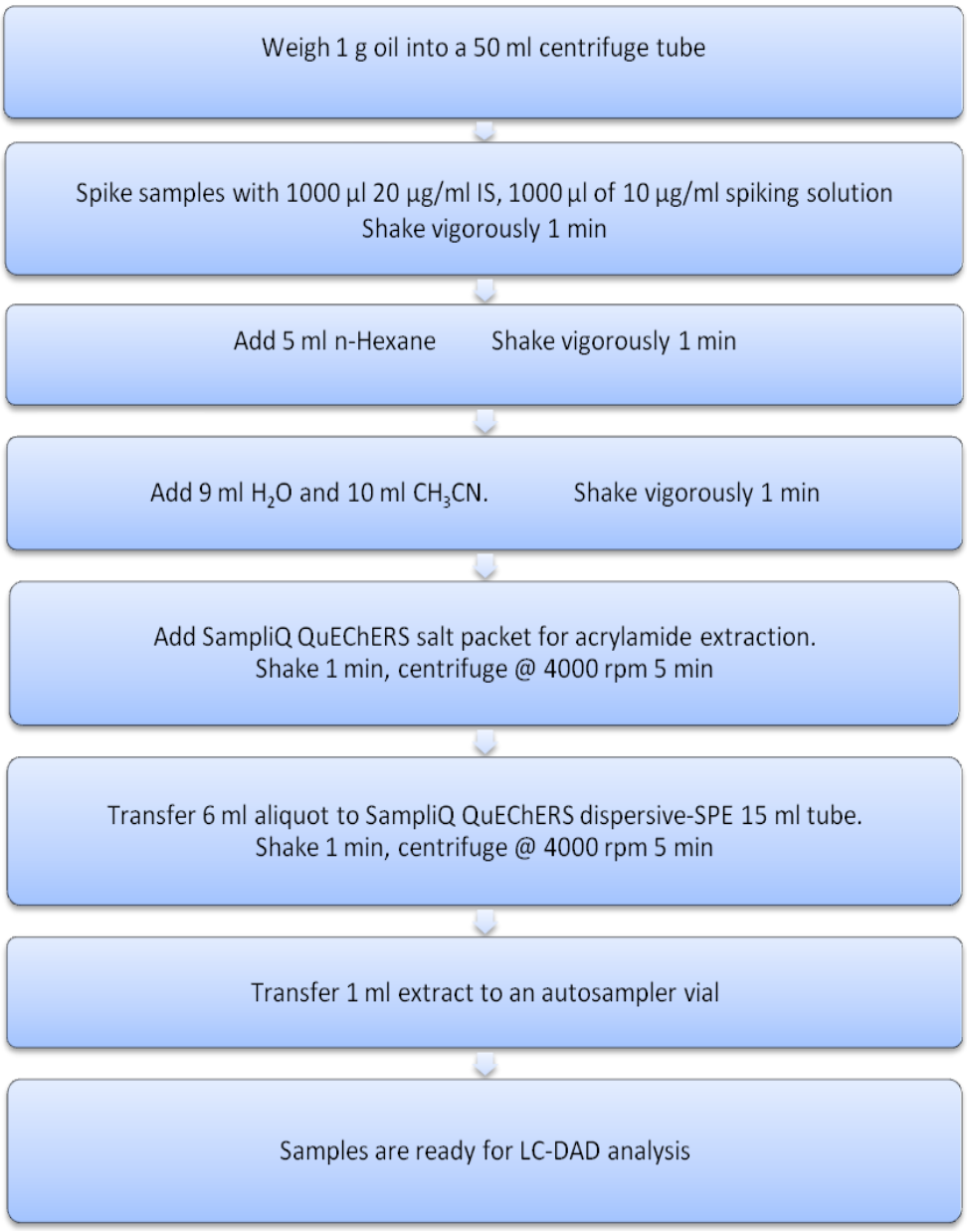
### *4.5.6 Sample preparation for acrylamide in cooking oil*

***Extraction***

1 g cooking oil was placed into a 50 ml centrifuge tube from the SampliQ QuEChERS Extraction kit. Samples were spiked appropriately to yield working solutions for recoveries and reproducibility studies. Samples, in exception of the blank, were fortified with 1000 µl spiking solution and mixed with 9 ml water. After shaking vigorously for 1 min, 10 ml $CH_3CN$ was added, followed by an addition of Agilent SampliQ QuEChERS extraction salt mixture for acrylamides (p/n 5082-5850). The QuEChERS extraction packet contained 4 g of anhydrous $MgSO_4$ and 0.5 g NaCl. The sample tubes were hand shaken vigorously for 1 min and then centrifuged at 4000 rpm for 5 min.

***Dispersive SPE clean-up***

6 ml of the upper ACN layer were transferred into a SampliQ QuEChERS AOAC Dispersive SPE 15 ml tube. This SPE tube contained 400 mg of PSA and 1200 mg of anhydrous $MgSO_4$. The tubes were then further centrifuged at 4000 rpm for 5 min. 1000 µl extract were placed in an autosampler vial for an HPLC-DAD analysis. Flow chart for the QuEChERS sample preparation procedure for acrylamides in cooking oil is depicted in Scheme 4.5.6.




**Scheme 4.5.6: QuEChERS Flow chart for acrylamide in cooking oil**

# CHAPTER 5

# Results and Discussions

## 5.1 Acidic, basic and neutral drugs in urine and plasma matrices

An isocratic reversed phase HPLC method was developed for the separation of analytes in the urine and plasma samples. Different chromatographic conditions (columns, gradients, mobile phases and flow rates) were investigated to optimize the separation of analysed drugs in the shortest time. The buffer concentration (25 mM) was low enough to minimize the abrasive effect on the pump seals, consistent to previous results [191]. The analytes were well separated, with good peak resolutions; sharpness and symmetry. Typical chromatograms of a standard mixture containing secobarbital (10 µg/ml) nortriptyline (5 µg/ml), ketoprofen (5 µg/ml) and naproxen (2 µg/ml) for urine analysis under conditions shown in Table 4.4.1 and standard mixture (7 µg/ml) of acetaminophen, amphetamine, ranitidine (IS), p-toluamide, m-toluidine and Phenobarbital (plasma analysis) under chromatographic conditions shown in Table 4.4.2 are shown in Fig. 5.1.1 (A) and (B) respectively.

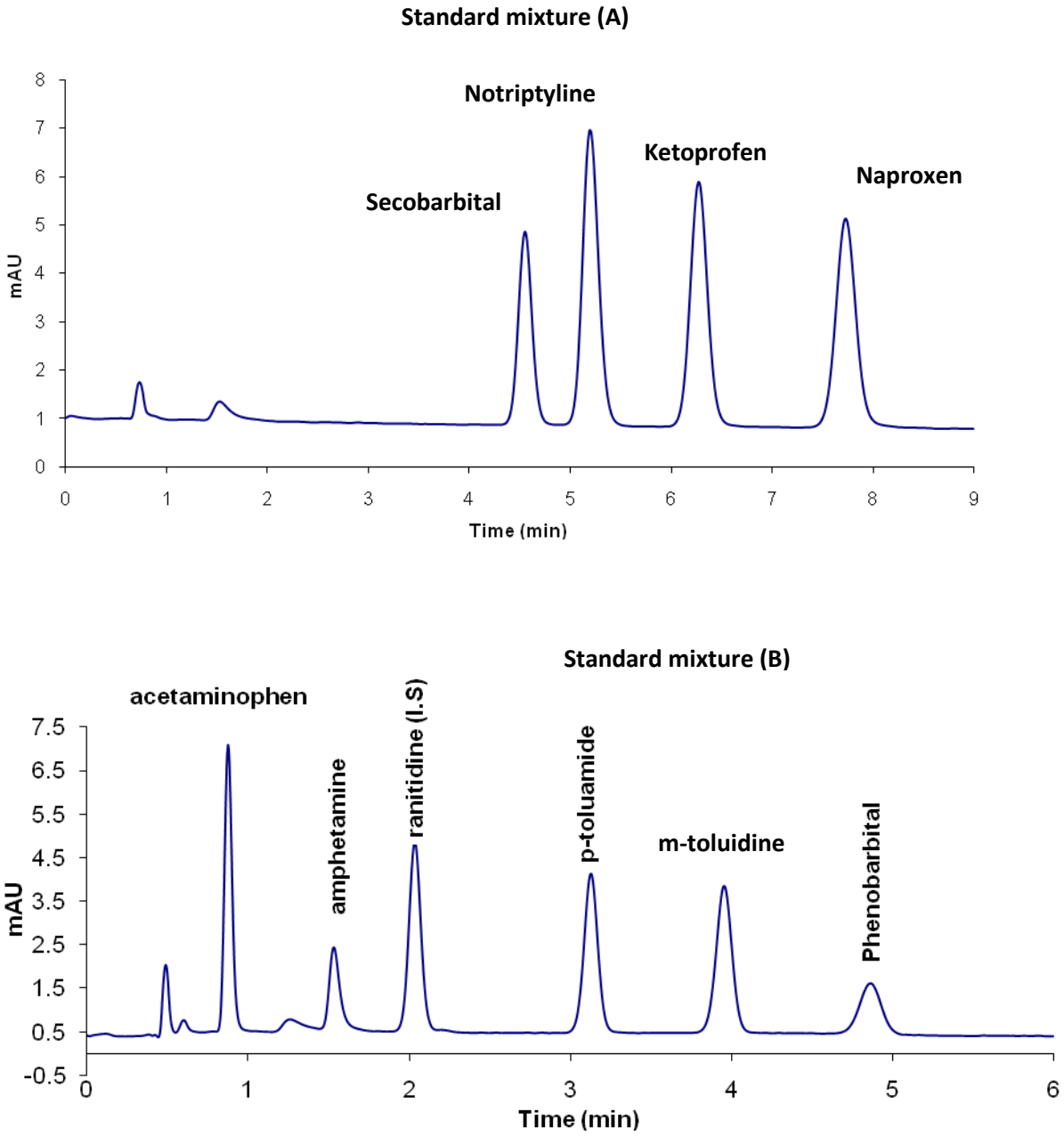



**Figure 5.1.1: Typical RP-HPLC-DAD chromatograms of standard mixtures: (A) drugs in urine; secobarbital (10 µg/ml) nortriptyline (5 µg/ml), ketoprofen (5 µg/ml) and naproxen (2 µg/ml): (B) drugs in plasma (7 µg/ml).**

Due to the diverse polarities and pH characters of the compounds used each one of them was monitored at its maximum absorption wavelength (Table 5.1.1). Secobarbital gave a weak response compared to other drugs in the standard mixture and its concentration was adjusted upward to provide a stronger signal.

**Table 5.1.1: Chemical and physical characteristics of the drugs in urine**

| Drug | Classification | Log P | pKa | $\Lambda_{max}$ (nm) |
|---|---|---|---|---|
| Secobarbital | Neutral | 1.97 | 7.90 | 222 |
| Nortriptyline | Basic | 4.28 | 9.70 | 242 |
| Ketoprofen | Acidic | 0.97 | 5.94 | 258 |
| Naproxen | Acidic | 3.18 | 4.53 | 230 |

In order to enhance method selectivity and prolong the column lifetime matrices, human plasma and urine, were hydrolysed. The samples were hydrolysed by either an acid or base hence they were deprotonated. Mixed-mode cation- and anion-exchange SPE columns were employed, separately, in the extraction method. SampliQ SAX, a polymeric mixed-mode strong anion, exchanger and SampliQ SCX, a mixed-mode strong cation exchange sorbent were successfully employed. The acidic, basic and neutral drugs from a spiked urine and plasma samples were simultaneously extract using the SPE procedures shown in Scheme 4.5.1 and 4.5.2 respectively. The undissociated compounds were retained in the hydrophobic portion of the sorbents and eluted in the neutral fractions. The ionised drugs were retained by either the strong anion or cation exchange interactions with the sorbent and eluted in the acidified or ammoniated fraction.

The chromatograms of a blank urine extract from a healthy volunteer and of the spiked urine extract are shown in Fig. 5.1.3 (a) and (b), respectively. The neutral (secobarbital) and basic (nortriptyline) drugs were eluted in the neutral fraction (Fig. 5.1.3b) as they were retained through hydrophobic interactions. Chromatograms of the acidic fractions, the urine blank and spiked urine extract are shown in Fig 5.1.3 (c) and (d) respectively. The acidic drugs (naproxen and ketoprofen) were retained by the strong anion exchange functionalities of the sorbent. However, traces (< 10%) of the neutral/basic drugs could be seen in the acidic fraction. A larger volume of methanol in the prior step (first elution) could have been used to improve extraction efficiency. As can be seen {Fig. 5.1.3 (a) and (c)}, no interference from the matrix were present and all peaks were still well resolved.

For the spiked plasma samples, the neutral and acidic drugs were eluted in the neutral fraction (see Fig. 5.1.4 b); they were retained through hydrophobic interactions. The basic drugs were retained by the strong cation exchange functionalities of the sorbent and eluted separately in the basic fraction, as shown in Fig. 5.1.4 (d). As with the SAX sorbent, traces (< 10%) of the neutral/acidic drugs were found in the basic fraction. Similarly, an increase in the methanol volume could have improved the extraction efficiency.

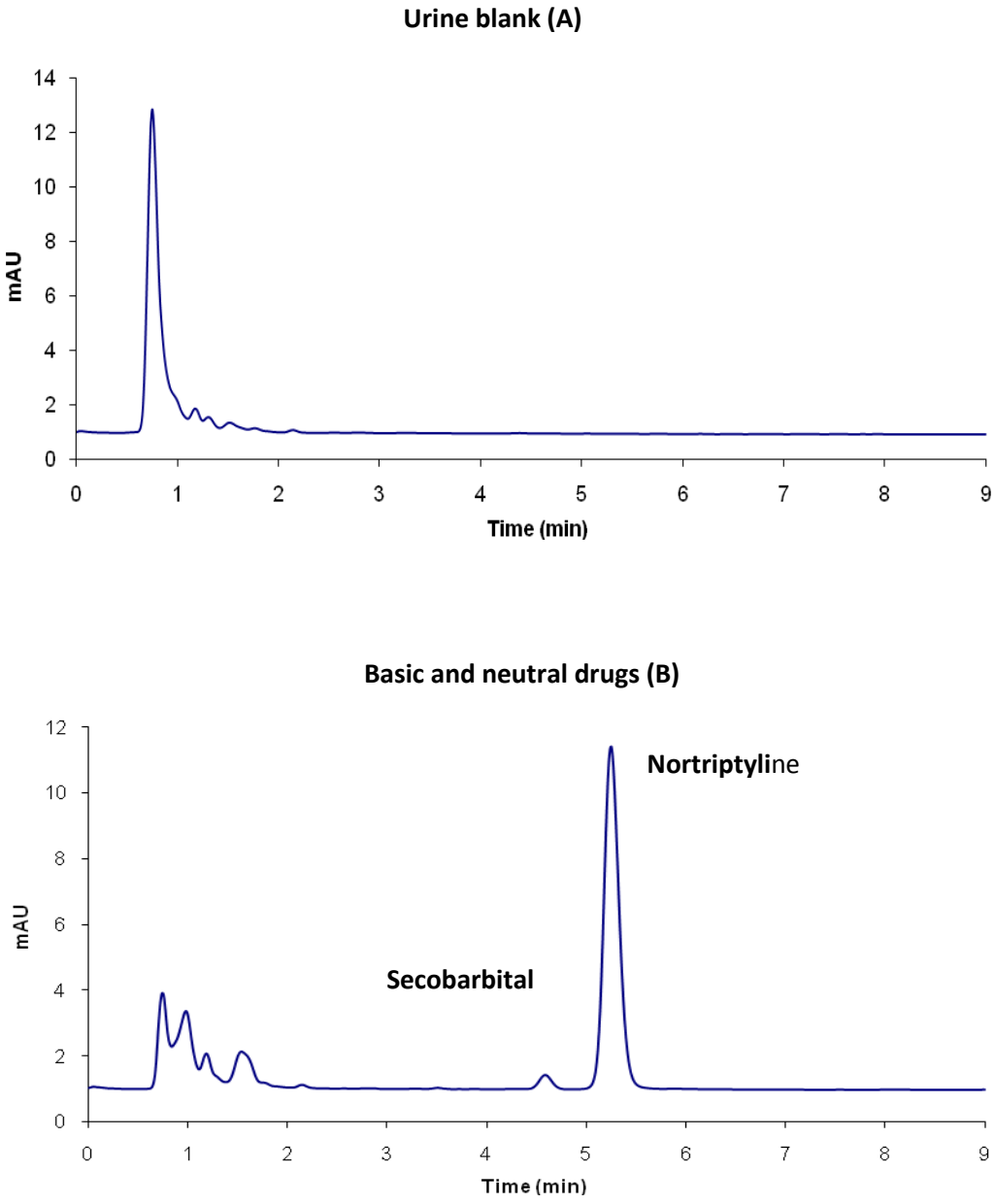



**Figure 5.1.3: Eluate 1 chromatograms of (a) blank urine sample; (b) spiked urine sample**

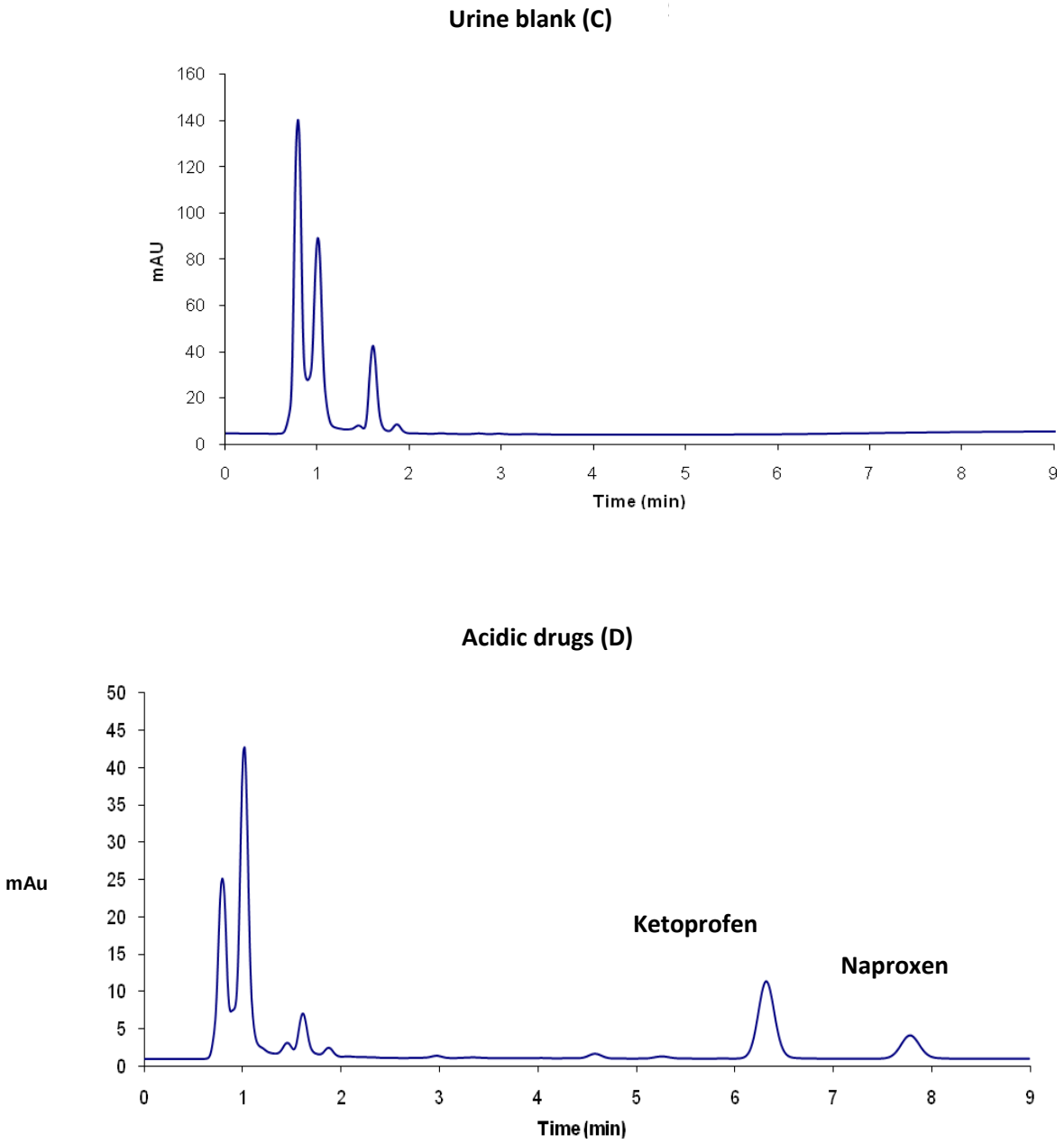



**Fig. 5.1.3: (continuation) Eluate 2 Chromatograms of (c) blank urine sample; (d) spiked urine sample; *Chromatographic conditions*: stationary phase, ZORBAX $C_{18}$ column (4.6 mm × 75 mm i.d., 3.5 µm); mobile phase, methanol /25 mM phosphate buffer containing pH 2.7; flow rate, 1.5 ml/min; injection volume, 5 µL; detection wavelength, 222 nm.**

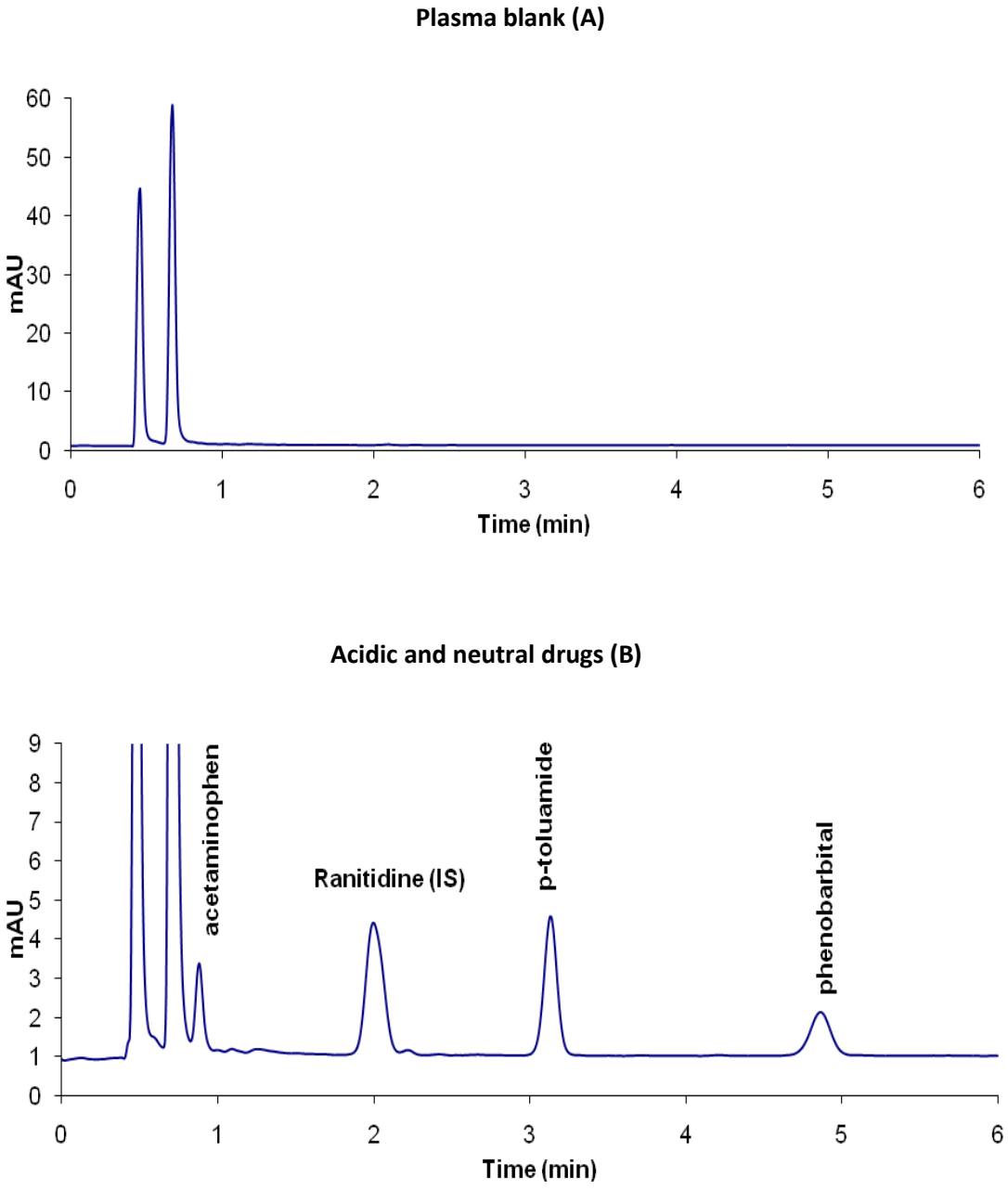



**Figure 5.1.4: Eluate 1 chromatograms of (a) blank plasma extract; (b) spiked plasma extract**

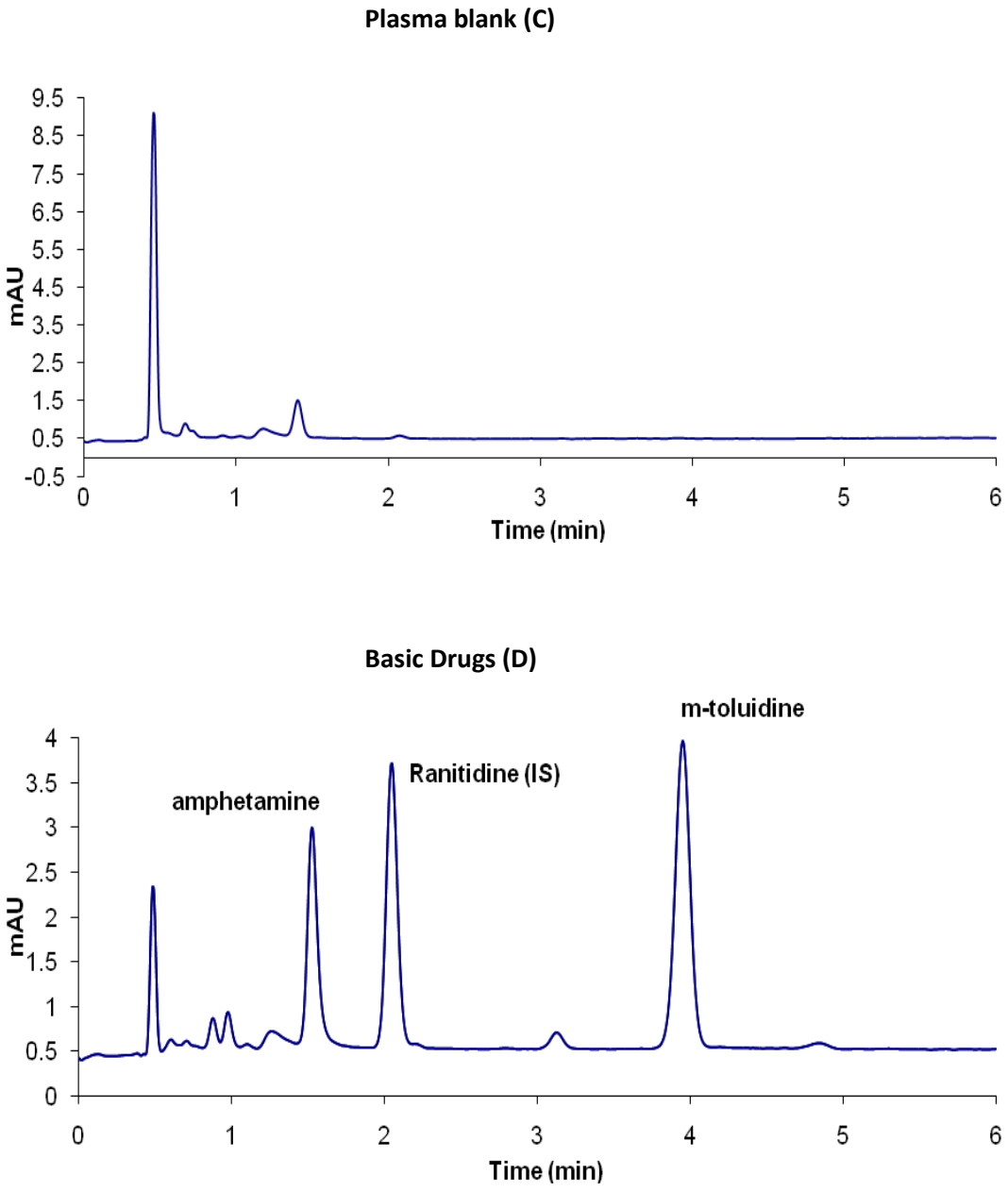



**Fig. 5.1.4: (continuation) Eluate 2 Chromatograms of (c) blank plasma extract; (d) spiked plasma extract; *Chromatographic conditions*: stationary phase, ZORBAX $C_{18}$ column (4.6 mm × 75 mm i.d., 3.5 µm); mobile phase, methanol /25 mM phosphate buffer containing pH 7; flow rate, 1.5 ml/min; injection volume, 5 µL; detection wavelength, 210 nm.**

Aliquots of working solutions at seven different concentrations, containing the IS at a constant concentration 50 µl for plasma analysis, were added to the urine or plasma blank. The resulting concentration ranges in urine were 0 - 8 µg/ml for nortriptyline and ketoprofen, 0 - 7 µg/ml for naproxen and 0 - 35 µg/ml for secobarbital. For plasma analysis, the concentration range was 0 – 10 µg/ml for all the analytes. Calibration curves were produced by plotting the analyte/IS peak area ratios against the corresponding concentrations of the analytes. Good linearity ($r^2 > 0.9990$) was attained for all the analytes in exception of ketoprofen and naproxen which showed linearity from 0 – 4.5 µg/ml. Linearity parameters are shown in Tables 5.1.2 and 5.1.3

**Table 5.1.2: Linearity parameters (urine analysis)**

| Drugs | Linear equation | Correlation coefficient ($r^2$) |
|---|---|---|
| **Secobarbital** | y = 1.5841x | $r^2 = 0.9994$ |
| **Nortriptyline** | y = 19.065x | $r^2 = 0.9996$ |
| **Ketoprofen** | y = 12.505x | $r^2 = 0.9994$ |
| **Naproxen** | y = 61.461x | $r^2 = 0.9996$ |

**Table 5.1.3: Linearity of amphetamine, acetaminophen, p-toluamide, m-toluidine and Phenobarbital in plasma**

| Analyte | Regression Equation | $R^2$ |
|---|---|---|
| Acetaminophen | Y = 0.0692x | 0.9993 |
| Amphetamine | Y = 0.0699x | 0.9994 |
| p- Toluamide | Y = 0.1145x | 0.9992 |
| m-Toluidine | Y = 0.1842x | 0.9991 |
| Phenobarbital | Y = 0.1072x | 0.9995 |

The recoveries were calculated by comparing the peak area of the analyte concentration in the spiked samples (urine and plasma) after SPE to that of the standard solution at the same concentration level. To demonstrate reproducibility the samples were analysed at three different concentration levels (n = 6). High recoveries (> 85%) were obtained with RSD values ranged from 0.06 to 1.12 for n = 6 runs (Figs. 5.1.5 and 5.1.6).

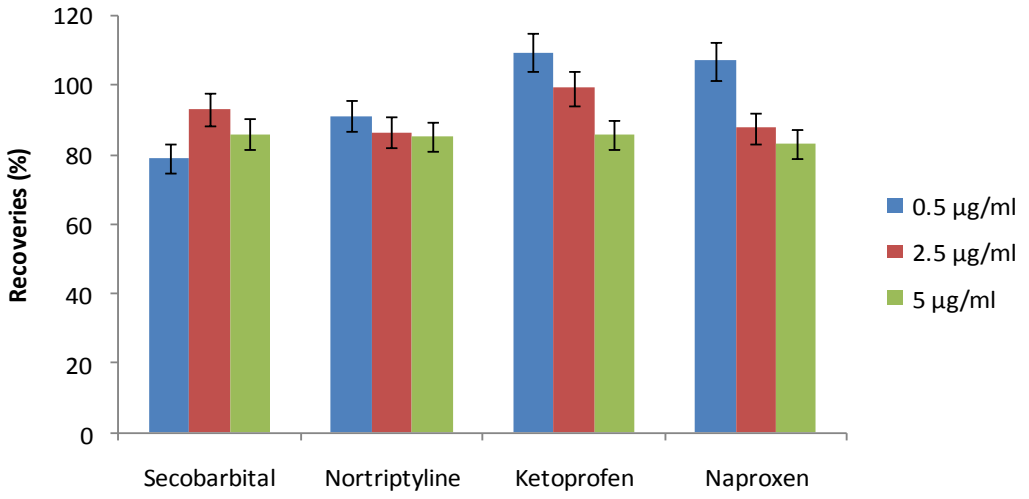



**Figure 5.1.5: Recoveries for Secobarbital, Nortriptyline, Ketoprofen and Naproxen from urine**

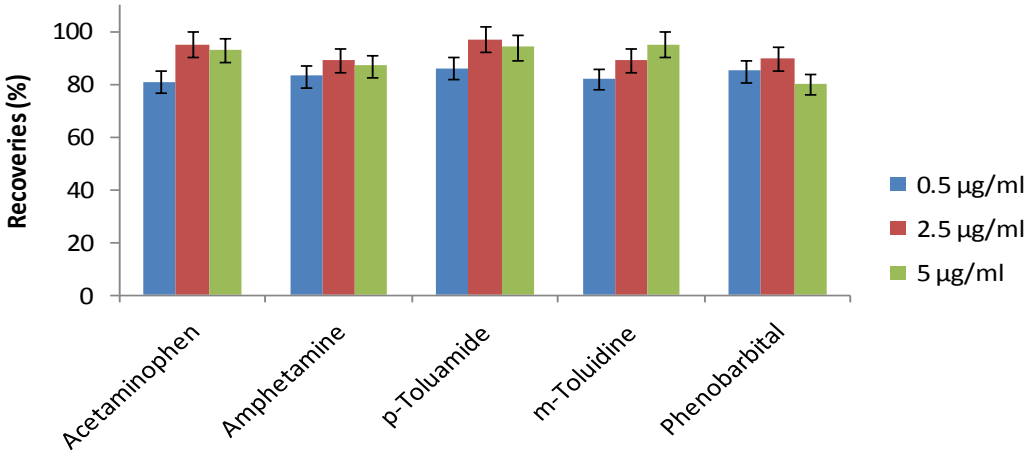



**Figure 5.1.6: Recoveries for Amphetamine, Acetaminophen, p-Toluamide, m-Toluidine and Phenobarbital in plasma**

Equations (5.1) and (5.2) were used to calculate LOD and LOQ, where $S_{yx}$ = standard error of the regression line and b = gradient (see Tables 5.1.4 and 5.1.5)

$$LOD = \frac{3.3 x Syx}{b} \quad \ldots\ldots (5.1)$$

$$LOQ = \frac{10.0 x Syx}{b} \quad \ldots\ldots (5.2)$$

**Table 5.1.4: LOD and LOQ for the analytes in urine**

| Drug | LOD (µg/ml) | LOQ (µg/ml) |
|---|---|---|
| **Secobarbital** | 0.21 | 0.81 |
| **Nortriptyline** | 0.04 | 0.12 |
| **Ketoprofen** | 0.03 | 1.04 |
| **Naproxen** | 0.03 | 2.74 |

**Table 5.1.5: LOD and LOQ for the analytes in plasma**

| Drug | LOD (µg/ml) | LOQ (µg/ml) |
|---|---|---|
| **Acetaminophen** | 0.39 | 0.85 |
| **Amphetamine** | 0.71 | 1.87 |
| **p-Toluamide** | 0.66 | 0.70 |
| **m-Toluidine** | 0.35 | 1.06 |
| **Phenobarbital** | 0.82 | 1.89 |

The method linearity after SPE as well as the limits of detection (LOD) and quantification (LOQ) were determined. The urine samples were spiked in the concentration range 0-25 µg/ml for secobarbital and 0 – 10 µg/ml for nortriptyline, ketoprofen and naproxen. These mixtures were subjected to the previously described SPE procedure (see Scheme 4.5.1) and injected into the HPLC system. The procedure was carried out in triplicate for each concentration.

Secobarbital and nortriptyline were linear in the chosen range while ketoprofen and naproxen showed linearity from 0 – 4.5 µg/ml. Table 5.1.6 shows the linearity equations and correlation coefficients.

**Table 5.1.6: Linearity of the method employing SPE**

| Drugs | Linear equation | Correlation coefficient ($r^2$) |
|---|---|---|
| **Secobarbital** | $y = 1.3325x$ | $r^2 = 0.9993$ |
| **Nortriptyline** | $y = 17.595x$ | $r^2 = 0.9991$ |
| **Ketoprofen** | $y = -1.2748x^2 + 17.896x$ | $r^2 = 0.9991$ |
| **Naproxen** | $y = -1.9003x^2 + 33.527x$ | $r^2 = 0.9993$ |

The plasma blanks spiked with the analytes at five different concentrations were subjected to the SPE procedure (Scheme 4.5.2). 50 µl of the internal standard was added and then each spiked plasma sample was prepared in triplicate. The analyte/IS peak area ratios were plotted against the corresponding concentrations. All the analytes were linear in the chosen concentration range (0 – 8 µg/ml) with $r^2 > 0.999$. Precision was determined by reproducibility studies expressed in percent relative standard deviations (% RSD) which were less than 10%. The analytical parameters for SCX SPE protocol are shown in Table 5.1.7.

**Table 5.1.7: Linearity of the method employing SPE**

| Parameter | SPE |
|---|---|
| Linearity | 0 – 8 |
| $R^2$ | 0.9990 – 0.9999 |
| % RSD | 1.10 – 6.05 |

## 5.2 Polycyclic Aromatic Hydrocarbons in fish and soil matrices

The analysis of organic and veterinary drug residue contaminants in food and environmental samples is usually hampered by interfering compounds present in these complex matrices. Therefore the challenge was to maximize recovery of analyte and minimize the accompanying interferences by proper extraction and clean-up procedures. The original QuEChERS method which used neutral extraction conditions [192] and the one that uses buffered acidic extraction conditions [193, 194] were adapted for this work. The recovery and reproducibility data demonstrate that neutral extraction conditions were effective for PAHs and acrylamide. The acidic extraction conditions were more effective for recovering sulfonamides. There are many different permutations of the QuEChERS approach which serve a useful purpose to improve results or practical efficiency for the given analyte (s)/matrix (es) applications [195]. The use of $CH_3CN$ as an extracting solvent eliminated the need to add co-solvents. High extraction yields for all the analytes, as shown by the recovery data, were attained (see Tables 5.2.2, 5.2.3, Fig. 5.5.2 and 5.6.1).

Furthermore, $CH_3CN$ solvent was compatible with the HPLC – FLD/DAD procedures employed for PAHs and acrylamide. Therefore no evaporation or reconstitution solvent was required. This was particularly important as acrylamide and some of the extremely volatile PAHs (naphthalene, acenaphthene and fluorene) may have been lost during an evaporation step. In addition, $CH_3CN$ is immiscible with hexane and this resulted in a simple clean-up protocol. The analytes were extracted into a water soluble solvent (neutral $CH_3CN$ or 1% (v/v) acetic acid in $CH_3CN$). They were then partitioned into organic solvent in the presence of a salt mixture (salting out effect). The acetonitrile phase was further cleaned up and dried by mixing with the SPE sorbents and anhydrous $MgSO_4$. The QuEChERS method employed PSA sorbent for d-SPE. PSA is a weak anion exchanger which strongly interacts with polar organic acids, sugars and fatty acids. The addition of acid in the $CH_3CN$ partitioning step impeded the performance of PSA in the dispersive step preventing the loss of analytes [196].

The separation of the 16 PAHs was attained on a PAH $C_{18}$ column (4.6 mm x 50 mm, 1.8 µm) by gradient elution with a binary system of acetonitrile – water. The chromatograms for the standard mixture at level 1concentrations of PAHs (see Table 5.2.1) are presented in Fig. 5.2.1 while Fig. 5.2.2 (a) and (b) for the blank fish extract and overlay chromatograms of the spiked fish; Fig 5.2.3 (c) and (d) represent soil blank extract and overlay chromatograms of the spiked soil extract respectively at level 1. The fluorescence detector was set at varying emission wavelengths (see Table 4.4.3) for detection and quantification to accommodate the diverse absorption intensities of the PAHs. However, due to lack of a flourophore, UV detection at 230 nm was employed for acenaphthylene.

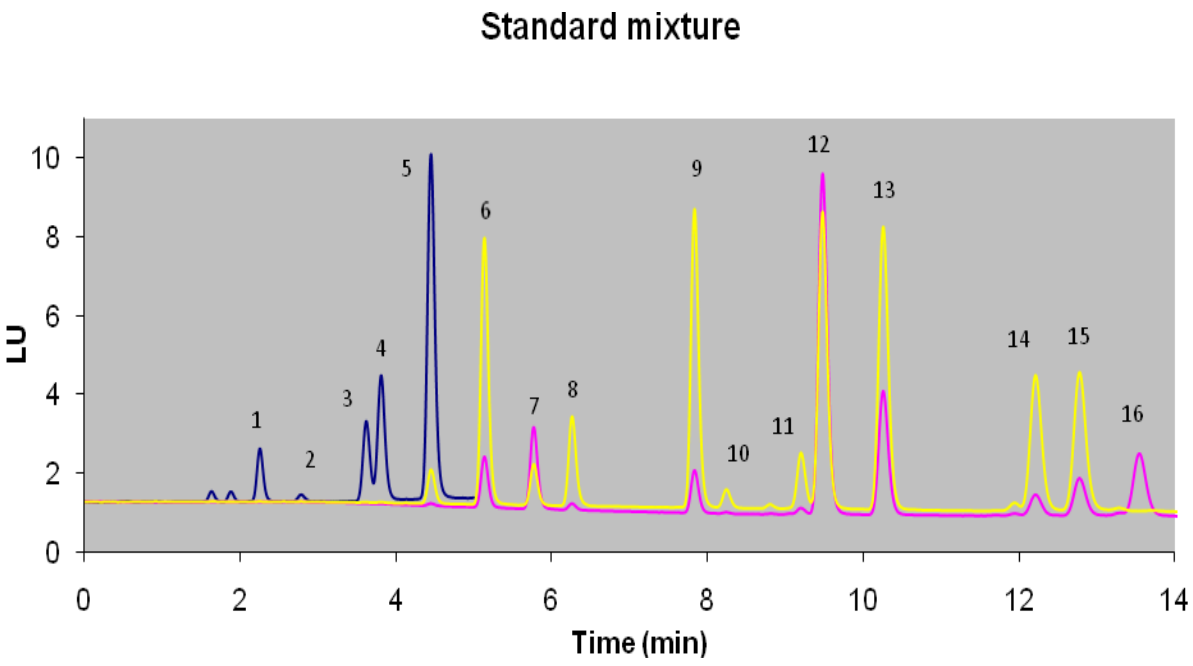



**Figure 5.2.1: Overlay HPLC – FLD chromatograms of the standard mixture containing: 1. Nap 2. Acy 3. Ace 4. Flu 5. Phe 6. Ant 7. Fln 8. Pyr 9. BaA 10. Chr 11. BeP 12. BeA 13. BkF 14. DahA 15. BghiP 16. InP**

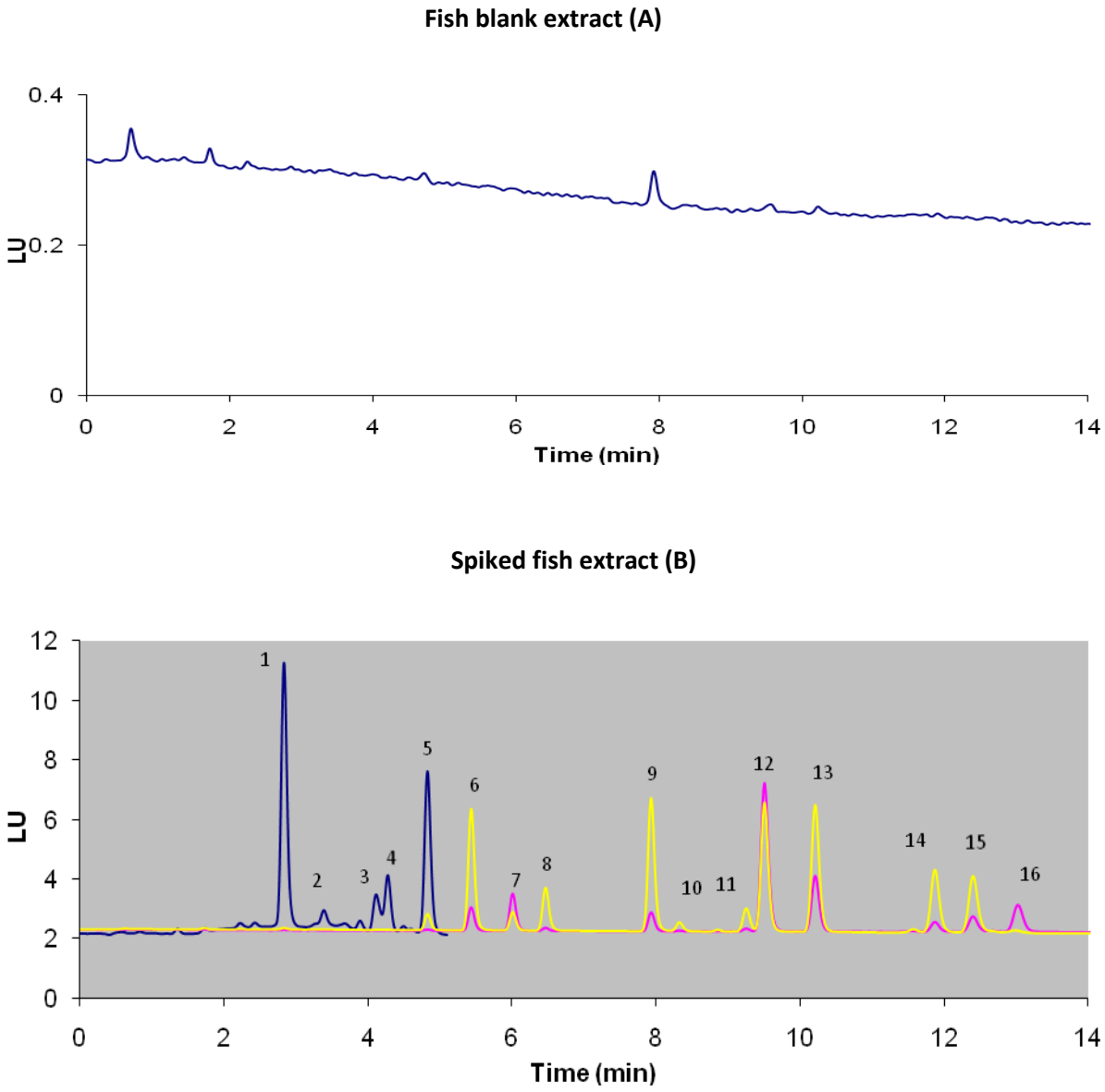



**Figure 5.2.2: (A) Chromatogram of the blank fish extract; (B) Overlay HPLC – FLD chromatograms of the spiked fish sample containing: 1. Nap 2. Acy 3. Ace 4. Flu 5. Phe 6. Ant 7. Fln 8. Pyr 9. BaA 10. Chr 11. BeP 12. BeA 13. BkF 14. DahA 15. BghiP 16. InP**

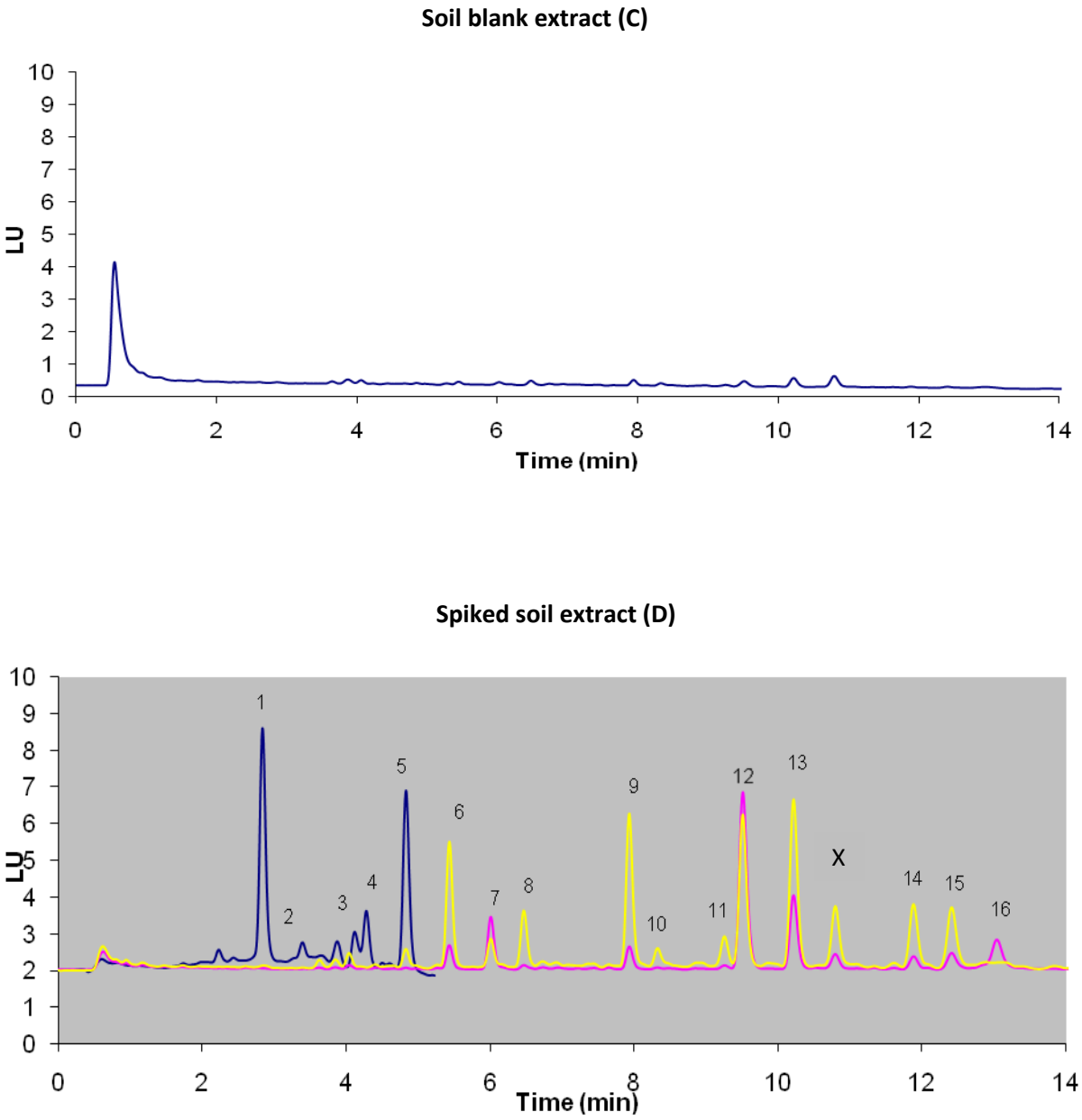



**Figure 5.2.3: (C) Chromatogram of the blank soil extract; (D) Overlay HPLC – FLD chromatograms of the spiked soil sample containing: 1. Nap 2. Acy 3. Ace 4. Flu 5. Phe 6. Ant 7. Fln 8. Pyr 9. BaA 10. Chr 11. BeP 12. BeA 13. BkF 14. DahA 15. BghiP 16. InP X = unknown**

**Table 5.2.1: PAHs spiking levels (preparation of working standard solutions)**

| PAH | Spiking level (ng/g) | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| **Naphthalene** | 20 | 100 | 200 |
| ***Acenaphthylene** | 20 | 100 | 200 |
| **Acenaphthene** | 10 | 50 | 100 |
| **Fluorene** | 10 | 50 | 100 |
| **Phenanthrene** | 10 | 50 | 100 |
| **Anthracene** | 10 | 50 | 100 |
| **Fluoranthene** | 10 | 50 | 100 |
| **Pyrene** | 10 | 50 | 100 |
| **1,2-Benzanthracene** | 5 | 20 | 50 |
| **Chrysene** | 10 | 50 | 100 |
| **Benzo[e]pyrene** | 5 | 20 | 50 |
| **Benz[e]acenaphthylene** | 5 | 20 | 50 |
| **Benzo[k]fluoranthene** | 5 | 20 | 50 |
| **Dibenzo[a,h]anthracene** | 5 | 20 | 50 |
| **Benzo[g,h,i]perylene** | 5 | 20 | 50 |
| **Indeno[1,2,3-cd]pyrene** | 5 | 20 | 50 |

* UV detection at 230 nm

**Table 5.2.2: Recovery and repeatability for PAHs in spiked fish sample (n = 6)**

| PAH | Level of spiking (ng/g) (n = 6) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | |
| | %Rec | %RSD | %Rec | %RSD | %Rec | %RSD |
| **Naphthalene** | 94.7 | 1.4 | 97.9 | 1.1 | 93.8 | 1.4 |
| ***Acenaphthylene** | 87.8 | 1.7 | 96.3 | 1.2 | 85.6 | 0.8 |
| **Acenaphthene** | 92.1 | 1.5 | 93.0 | 1.8 | 96.7 | 0.8 |
| **Fluorene** | 98.1 | 1.5 | 89.9 | 1.0 | 97.2 | 0.9 |
| **Phenanthrene** | 90.6 | 0.9 | 93.8 | 0.8 | 83.1 | 1.7 |
| **Anthracene** | 96.7 | 1.0 | 87.6 | 0.8 | 92.1 | 0.6 |
| **Fluoranthene** | 83.4 | 1.3 | 93.9 | 1.5 | 95.9 | 1.2 |
| **Pyrene** | 93.5 | 1.8 | 86.1 | 1.3 | 95.0 | 1.4 |
| **1,2-Benzanthracene** | 94.5 | 1.3 | 89.6 | 1.6 | 94.9 | 1.0 |
| **Chrysene** | 101.0 | 1.4 | 97.8 | 1.7 | 87.2 | 1.6 |
| **Benzo[e]pyrene** | 88.8 | 1.5 | 85.2 | 1.9 | 95.0 | 1.4 |
| **Benz[e]acenaphthylene** | 95.5 | 0.7 | 92.7 | 0.7 | 89.2 | 0.9 |
| **Benzo[k]fluoranthene** | 93.5 | 0.8 | 94.6 | 0.9 | 98.9 | 0.8 |
| **Dibenzo[a,h]anthracene** | 88.2 | 0.9 | 97.3 | 1.1 | 97.1 | 0.6 |
| **Benzo[g,h,i]perylene** | 98.4 | 0.8 | 95.5 | 1.6 | 98.2 | 0.7 |
| **Indeno[1,2,3-cd]pyrene** | 91.5 | 1.5 | 97.9 | 0.9 | 94.3 | 0.7 |

* UV detection at 230 nm  Rec = recovery

**Table 5.2.3: Recoveries and RSDs for the sixteen polycyclic aromatic hydrocarbons in soil sample (n = 6)**

| PAH | Level of spiking (ng/g) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | |
| | %Rec | %RSD | %Rec | %RSD | %Rec | %RSD |
| **Naphthalene** | 96.5 | 0.7 | 86.2 | 1.4 | 92.8 | 1.4 |
| ***Acenaphthylene** | 87.3 | 0.7 | 90.0 | 1.3 | 91.7 | 1.6 |
| **Acenaphthene** | 91.0 | 1.8 | 89.2 | 1.1 | 89.7 | 1.4 |
| **Fluorene** | 95.2 | 0.8 | 91.4 | 1.3 | 86.0 | 1.2 |
| **Phenanthrene** | 93.0 | 1.0 | 94.6 | 0.7 | 98.1 | 0.9 |
| **Anthracene** | 91.9 | 1.1 | 90.0 | 0.8 | 97.6 | 0.7 |
| **Fluoranthene** | 93.5 | 1.7 | 94.7 | 1.3 | 87.9 | 1.5 |
| **Pyrene** | 96.3 | 1.3 | 89.4 | 0.9 | 91.2 | 1.9 |
| **1,2-Benzanthracene** | 92.9 | 1.7 | 87.8 | 1.5 | 92.8 | 0.7 |
| **Chrysene** | 98.0 | 1.4 | 92.4 | 1.2 | 95.8 | 1.0 |
| **Benzo[e]pyrene** | 97.2 | 1.0 | 97.5 | 0.7 | 90.3 | 0.8 |
| **Benz[e]acenaphthylene** | 93.2 | 0.9 | 93.1 | 0.6 | 98.0 | 0.7 |
| **Benzo[k]fluoranthene** | 94.1 | 1.1 | 97.6 | 0.7 | 91.4 | 1.1 |
| **Dibenzo[a,h]anthracene** | 89.2 | 1.0 | 99.2 | 1.7 | 90.8 | 1.3 |
| **Benzo[g,h,i]perylene** | 91.0 | 0.9 | 96.7 | 0.8 | 97.3 | 1.6 |
| **Indeno[1,2,3-cd]pyrene** | 86.0 | 1.2 | 97.8 | 0.8 | 94.3 | 1.3 |

* UV detection at 230 nm  Rec = recovery

The linearity of the PAHs method was determined by extracting samples spiked at concentration range of 0 – 300 ng/g. The linear calibration curves were obtained by plotting the peak area for each analyte versus its concentration. All the analytes were linear in the chosen concentration range with $r^2 > 0.9990$.

The limits of detection and quantification were estimated from the concentration of analytes of interest required to give a signal-to-noise ratio of 3 and 10 respectively. Tables 5.2.4 and 5.2.5 show the regression equations, correlation coefficients, limits of detection and quantification for analysis in fish and soil respectively.

**Table 5.2.4: Linearity, LOD and LOQ for the sixteen polycyclic aromatic hydrocarbons (Fish)**

| PAH | Regression equation | $R^2$ | LOD | LOQ |
|---|---|---|---|---|
| **Naphthalene** | Y = 0.0222x + 0.1366 | 0.9991 | 0.62 | 2.07 |
| ***Acenaphthylene** | Y = 0.0544x – 0.0130 | 0.9993 | 0.25 | 0.83 |
| **Acenaphthene** | Y = 0.0184 x – 0.0204 | 0.9998 | 0.56 | 1.87 |
| **Fluorene** | Y = 0.0323x – 0.1717 | 0.9990 | 0.12 | 0.40 |
| **Phenanthrene** | Y = 0.0950x + 0.0086 | 0.9995 | 0.18 | 0.60 |
| **Anthracene** | Y = 0.0838x – 0.1265 | 0.9991 | 0.24 | 0.80 |
| **Fluoranthene** | Y = 0. 0247x – 0.0237 | 0.9994 | 0.04 | 0.16 |
| **Pyrene** | Y = 0.0218x - 0.0432 | 0.9998 | 0.09 | 0.30 |
| **1,2-Benzanthracene** | Y = 0.0120x - 0.0103 | 0.9994 | 0.03 | 0.10 |
| **Chrysene** | Y = 0.0052x + 0.0086 | 0.9990 | 0.28 | 0.93 |
| **Benzo[e]pyrene** | Y = 0.0144x – 0.0037 | 0.9997 | 0.04 | 0.16 |
| **Benz[e]acenaphthylene** | Y = 0.1186x – 0.032 | 0.9995 | 0.07 | 0.23 |
| **Benzo[k]fluoranthene** | Y = 0.0464x + 0.0969 | 0.9997 | 0.05 | 0.16 |
| **Dibenzo[a,h]anthracene** | Y = 0.0531x + 0.0001 | 0.9990 | 0.84 | 2.80 |
| **Benzo[g,h,i]perylene** | Y = 0.0440x + 0.0722 | 0.9993 | 0.11 | 0.36 |
| **Indeno[1,2,3-cd]pyrene** | Y = 0.0324x – 0.0912 | 0.9993 | 0.05 | 0.18 |

* UV detection at 230 nm

**Table 5.2.5: Linearity, LOD and LOQ for the sixteen polycyclic aromatic hydrocarbons (Soil)**

| PAH | Regression equation | $R^2$ | LOD | LOQ |
|---|---|---|---|---|
| **Naphthalene** | Y = 0.0266x + 0.1568 | 0.9992 | 0.48 | 1.6 |
| ***Acenaphthylene** | Y = 0.0580x – 0.1323 | 0.9991 | 0.06 | 0.20 |
| **Acenaphthene** | Y = 0.0176 x + 0.0122 | 0.9995 | 0.12 | 0.41 |
| **Fluorene** | Y = 0.0358x – 0.1701 | 0.9991 | 0.24 | 0.79 |
| **Phenanthrene** | Y = 0.1097x - 0.4277 | 0.9994 | 0.07 | 0.22 |
| **Anthracene** | Y = 0.0884x – 0.096 | 0.9993 | 0.18 | 0.60 |
| **Fluoranthene** | Y = 0. 0273x – 0.0069 | 0.9997 | 0.07 | 0.24 |
| **Pyrene** | Y = 0.0284x - 0.1041 | 0.9993 | 0.005 | 0.02 |
| **1,2-Benzanthracene** | Y = 0.0120x - 0.0249 | 0.9994 | 0.78 | 0.26 |
| **Chrysene** | Y = 0.0067x + 0.0165 | 0.9992 | 0.007 | 0.02 |
| **Benzo[e]pyrene** | Y = 0.017x – 0.0252 | 0.9995 | 0.008 | 0.03 |
| **Benz[e]acenaphthylene** | Y = 0.1304x + 0.0727 | 0.9993 | 0.03 | 0.11 |
| **Benzo[k]fluoranthene** | Y = 0.052x + 0.0165 | 0.9993 | 0.06 | 0.21 |
| **Dibenzo[a,h]anthracene** | Y = 0.062x - 0.0346 | 0.9994 | 0.18 | 0.6 |
| **Benzo[g,h,i]perylene** | Y = 0.0599x + 0.0779 | 0.9995 | 0.18 | 0.81 |
| **Indeno[1,2,3-cd]pyrene** | Y = 0.0352x – 0.1588 | 0.9992 | 0.05 | 0.59 |

* UV detection at 230 nm

## 5.3 Sulfonamides in chicken muscles

Sulfonamides were derivatized in the pre-column mode with fluorescamine in acetone. The optimal incubation period was between 60 – 100 min and for reproducibility 60 min was the chosen time. The derivatised sulfonamides were detected with a single pair of wavelengths, $\lambda_{ex}$ = 405 nm and $\lambda_{em}$ = 495 nm. Fig. 5.3.1 shows a typical chromatogram of the standard mixture of the sulfonamides.

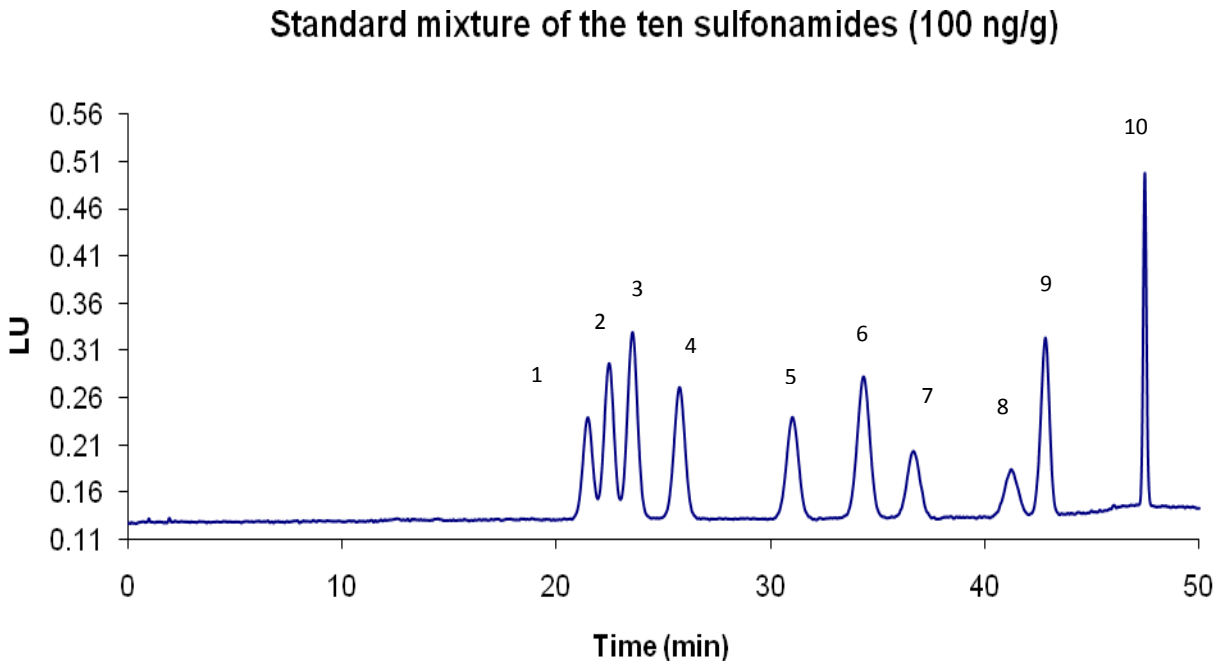



**Figure 5.3.1: Chromatogram of the standard mixture of the sulfonamides (100 ng/g): 1. Sulfadiazine 2. Sulfathiazole 3. Sulfapyridine (IS) 4. Sulfamerazine 5. Sulfamethazine 6. Sulfamethizole 7. Sulfamethoxypyridazine 8. Sulfachloropyridazine 9. Sulfamethoxazole 10. Sulfadimethoxine**

For the QuEChERS method, PSA sorbent has a strong interaction with polar organic acids, sugars and fatty compounds. However, it also reacted with the analytes of interest, sulfonamides, which resulted in very low recoveries. The method that employed acidified acetonitrile for extraction was therefore adapted [197]. The addition of the acid in the $CH_3CN$ partitioning step slowed down the performance of PSA in the dispersive step [198] and as a result the recoveries were tremendously improved.

Chromatograms of blank chicken muscle and spiked chicken muscle extract are shown in Fig.5.3.2 (a) and (b) respectively. The calibration curves of sulfonamides curves were obtained by plotting the relative responses of analytes (peak area of analyte / peak area of IS) to the relative concentration of analytes (concentration of analyte / concentration of IS). The curves were generated by spiking the sample blanks at a concentration range of 0 – 400 ng/g.

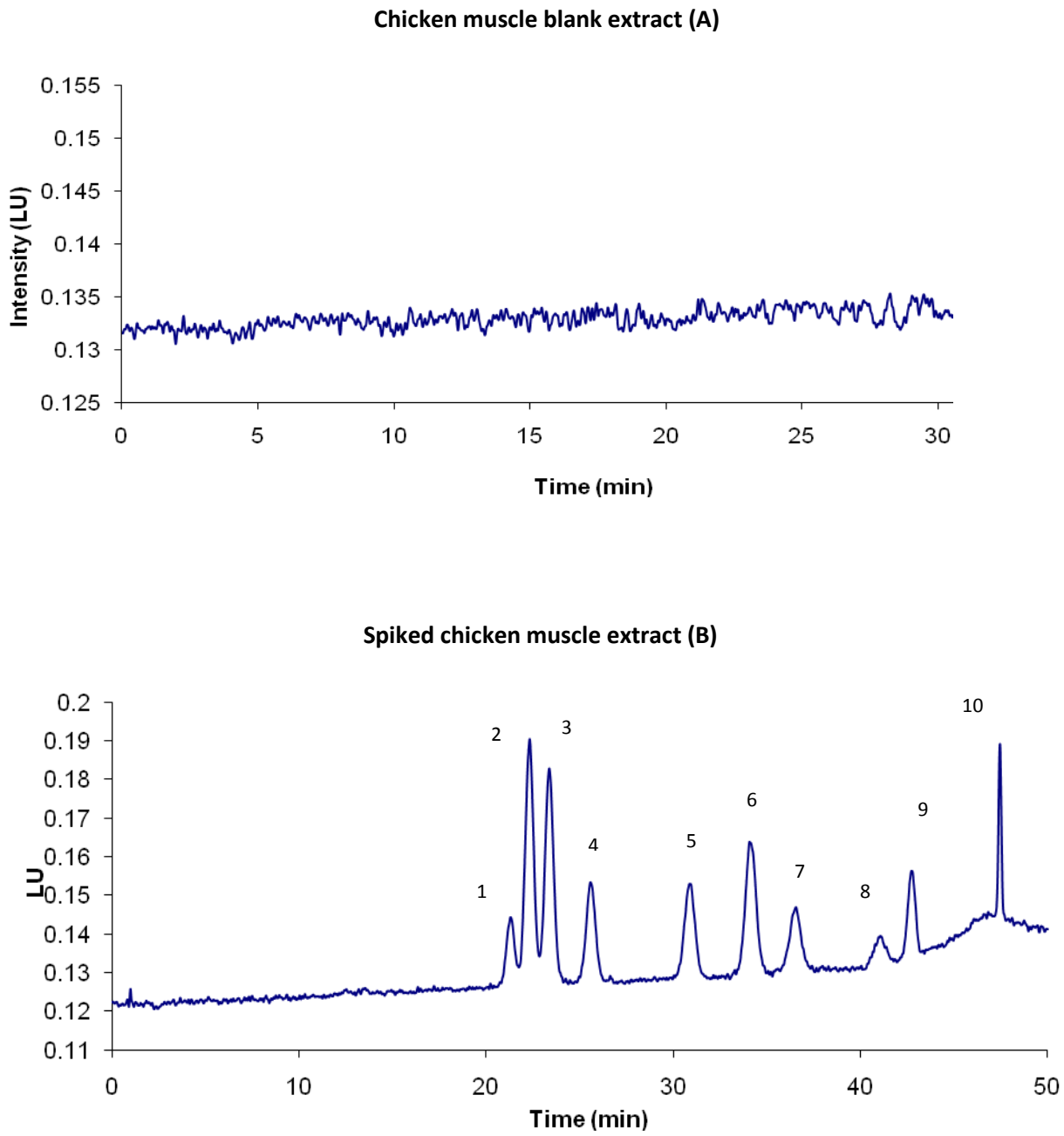



**Figure 5.3.2: Chromatograms of (A) blank chicken muscle; (B) spiked chicken muscle at 50 ng/g level: 1. Sulfadiazine 2. Sulfathiazole 3. Sulfapyridine (IS) 4. Sulfamerazine 5. Sulfamethazine 6. Sulfamethizole 7. Sulfamethoxypyridazine 8. Sulfachloropyridazine 9. Sulfamethoxazole 10. Sulfadimethoxine (see Fig. 5.3.4 for the mass spectra of these SAs)**

Linearity was attained for all sulfonamides with coefficients of regression > 0.9990 (see Table 5.3.1). The LODs ranged from 1.88 - 2.98 ng/g and were calculated at a signal-to-noise ratio (S/N) of 3. LOQs ranged from 6.27 - 9.93 ng/g and were calculated at S/N ration of 10.

**Table 5.3.1: Linearity, LOD and LOQ for the nine sulfonamides**

| Sulfonamide | Regression equation | $R^2$ | LOD | LOQ |
|---|---|---|---|---|
| **Sulfadiazine** | Y = 0.4154x + 0.0112 | 0.9995 | 2.00 | 6.67 |
| **Sulfathiozole** | Y = 1.0231x – 0.0757 | 0.9991 | 1.88 | 6.27 |
| **Sulfamerazine** | Y = 0.6735x + 0.0184 | 0.9993 | 2.49 | 8.30 |
| **Sulfamethazine** | Y = 0.6735x + 0.0042 | 0.9996 | 1.98 | 6.60 |
| **Sulfamethizole** | Y = 0.9751x + 0.0115 | 0.9995 | 2.30 | 7.67 |
| **Sulfamethoxypyridine** | Y = 0.4713x – 0.0069 | 0.9994 | 1.94 | 6.46 |
| **Sulfachloropyridazine** | Y = 0. 2769x + 0.0190 | 0.9992 | 1.88 | 6.27 |
| **Sulfamethoxazole** | Y = 0.6996x + 0.0421 | 0.9991 | 2.23 | 7.43 |
| **Sulfadimethoxine** | Y = 0.5008x + 0.0329 | 0.9991 | 2.98 | 9.93 |

Results shown in Fig. 5.3.3 indicate a high recovery range of 77.8 – 95.2% with relative standard deviations (RSD, *n* = 6) ranging from 1.5 to 4.7%. The chicken muscle samples were spiked at 100, 50 and 150 ng/g.

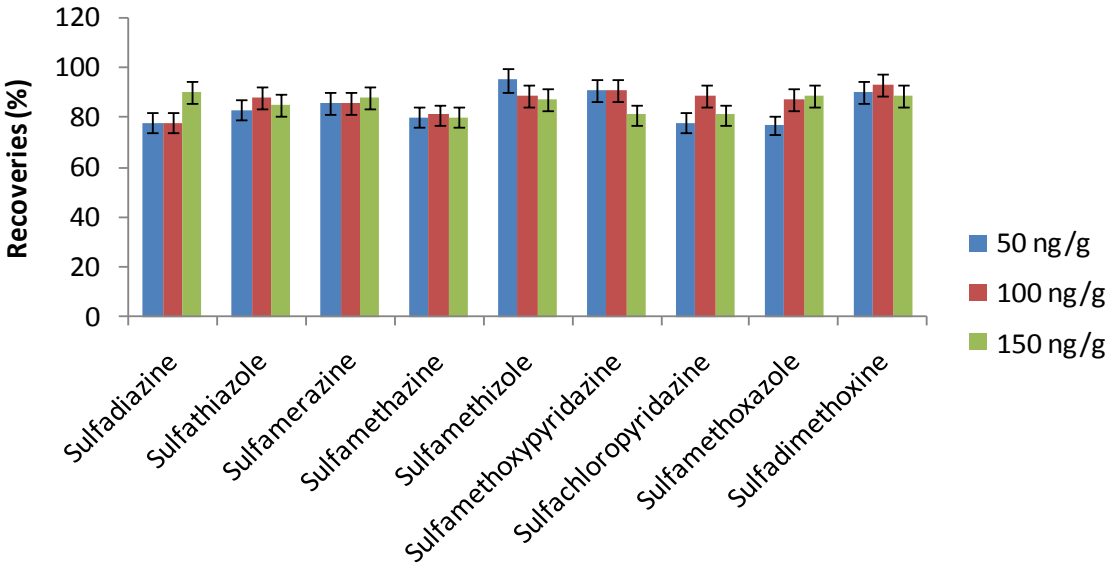



**Figure 5.3.3: Recoveries and RSDs (1.5 – 4.7%) for nine sulfonamides in chicken muscle (n = 6). These recoveries were evaluated on spiked chicken samples at MRL (100 ng/g), half MRL (50 ng/g) and one and a half MRL (150 ng/g).**

ESI–MS/MS conditions (see Table 4.4.4) were used to identify the sulfonamides. The precursor ion of each sulfonamide was selected for collision-induce dissociation (CID) experiment, which generated product ions. Consequently, the one product ion with high intensity, representing the characteristic of each compound, was set to monitor the identification. Figure 5.3.4 shows the mass spectra of sulfonamides (200 ng/g) in a spiked chicken muscle sample.

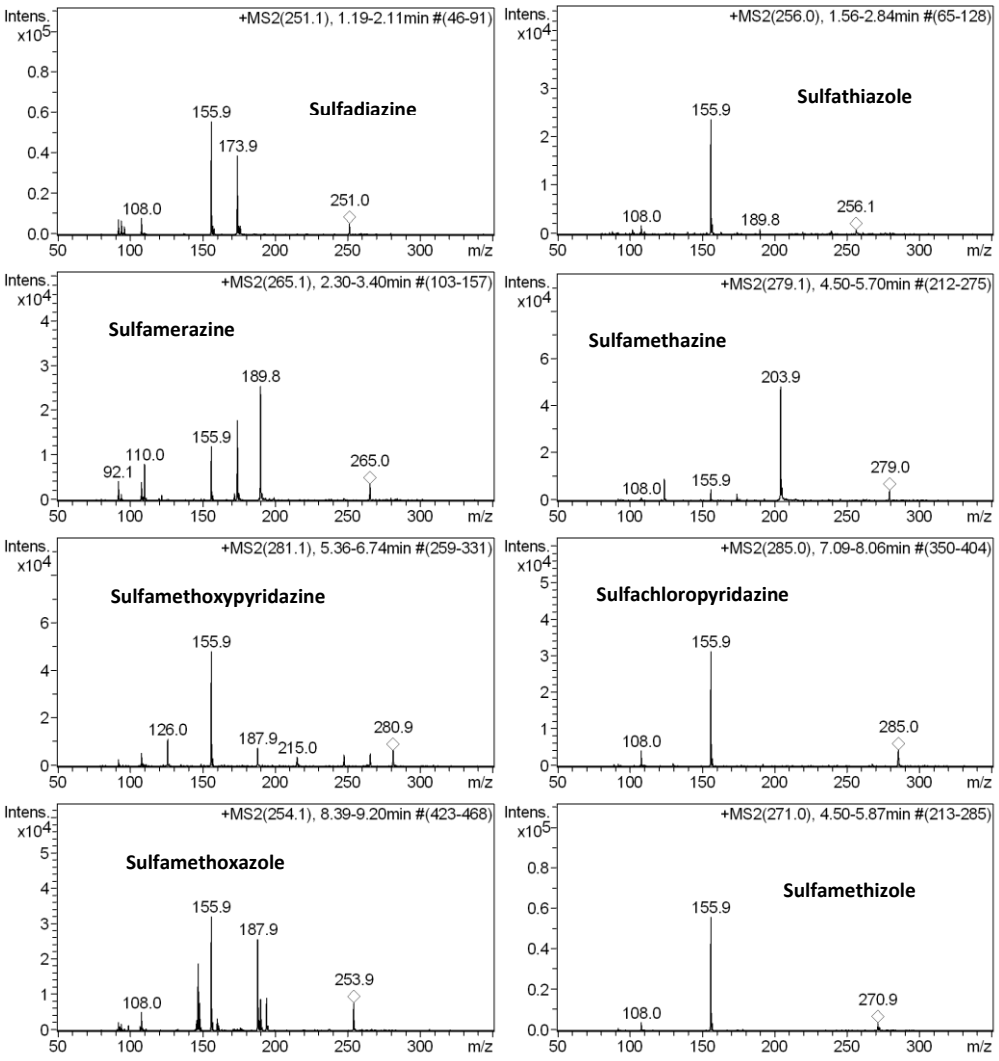



**Figure 5.3.4: Mass spectrum of chicken muscle sample spiked with sulfonamides (200 ng/g) using conditions described in Table 4.4.4**

MRM transition values used for the identification of sulfonamides are shown in Table 5.3.2. The molecular weights of the sulfonamides were concluded on the basis of their positive ion ESI mass spectra, which showed precursor ions $[M+H]^+$ in MS1. The characteristic fragment ions were used in MS2 for confirmation of the sulfonamides.

**Table 5.3.2: MS/MS conditions**

| $T_R$ (min) | Sulfonamide | Precursor ion | Product ions |
|---|---|---|---|
| 1.19 | Sulfadiazine | 251.0 | 155.9<br>108.0 |
| 1.56 | Sulfathiazole | 256.1 | 155.9<br>108.0 |
| 2.30 | Sulfamerazine | 265.0 | 189.8<br>155.9 |
| 4.50 | Sulfamethazine | 279.0 | 203.9<br>155.9 |
| 4.50 | Sulfamethizole | 270.0 | 155.9<br>108.0 |
| 5.36 | Sulfamethoxypyridazine | 280.9 | 155.9<br>126.0 |
| 7.09 | Sulfachloropyridiazine | 285.0 | 155.9<br>108.0 |
| 8.39 | Sulfamethozaxole | 253.9 | 155.9<br>108.0 |

## 5.4 Acrylamide in cooking oil

The separation of acrylamide and methacrylamide was attained on an Agilent ZORBAX HILIC Plus column (4.6 mm x 50 mm, 3.5 µm). An isocratic elution, with 3% 5 mM acetic acid and 97% acetonitrile mobile phase was employed. The column temperature was set at 30 °C with the flow rate maintained at 0.2 ml/min. Fig. 5.4.1 shows a typical chromatogram following the injection of the standard mixture. Different mobile phase polarity compositions, from 100% water to 100% acetonitrile, were evaluated. The best retention, with a shorter run time, was attained with 97% acetonitrile and 3% acetic acid.

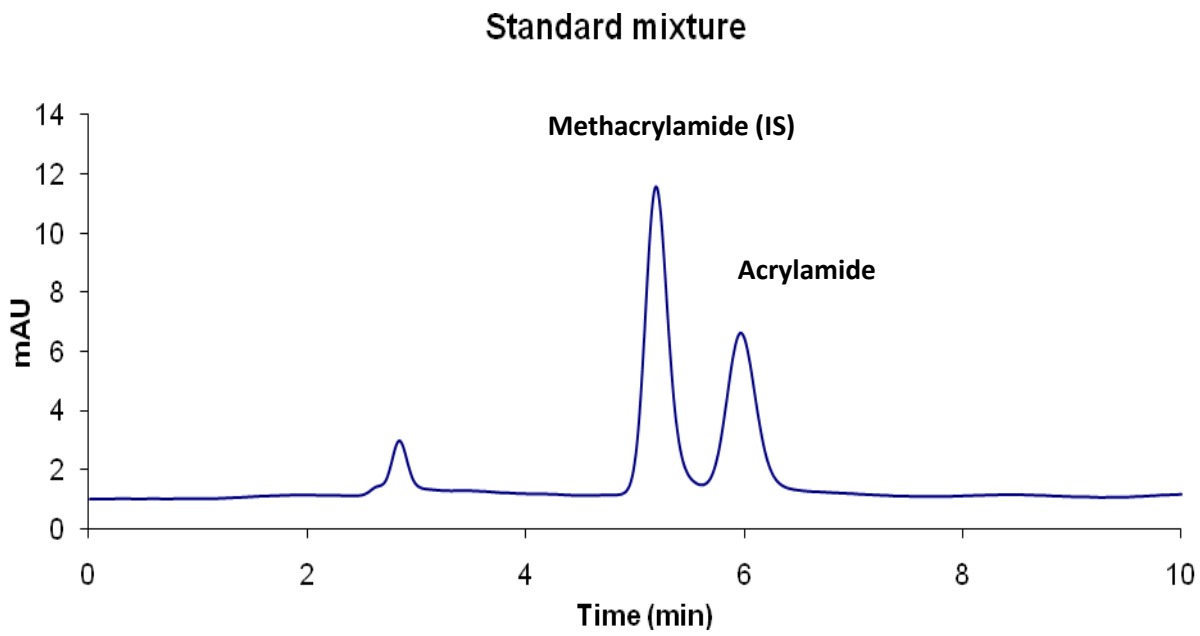



**Figure 5.4.1: Chromatogram of the standard mixture of acrylamide and methacrylamide (IS)**

A salt combination of 4 g $MgSO_4$ and 0.5 g NaCl was used to extract acrylamide from 1 g oil sample as well as to salt induce the acetonitrile – water phase separation. The samples were defattened with n-hexane to removed long chains of fatty acids that could create challenges in chromatographic. The fatty acid peaks usually overlap with the analyte or clogging the column [199]. The chromatograms of the oil blank and the spiked oil sample extract are shown in Fig. 5.4.2 (A) and (B) respectively. The blank oil extract did not show any detectable amounts of acrylamide. This was consistent to previous findings where the frying oils did not contain any detectable amounts of acrylamide (0.02 µg/ml LOD) prior to processing food [200].

A linear calibration curve (see Fig. 5.4.3) was obtained by plotting the relative responses of analyte (peak area of analyte / peak area of IS) to the relative concentration of analyte (concentration of analyte / concentration of IS). The curve was generated by spiking the sample blanks at a concentration range of 0 - 1500 ng/mL. Good linearity was demonstrated with $r^2$ = 0.9992. The LOD and LOQ were evaluated from the concentration of acrylamide required to give a signal- to-noise ratio of 3 and 10 respectively. The LOD was found to be 32.4 ng/mL while the LOQ was 108 ng/mL.

The recovery and reproducibility (RSD) were evaluated on spiked samples at three different fortification levels: 500, 1000, and 2000 ng/mL. The analysis was performed in replicates of six (n = 6) at each level. Fig. 5.4.4 shows the recoveries and RSD values for acrylamide.

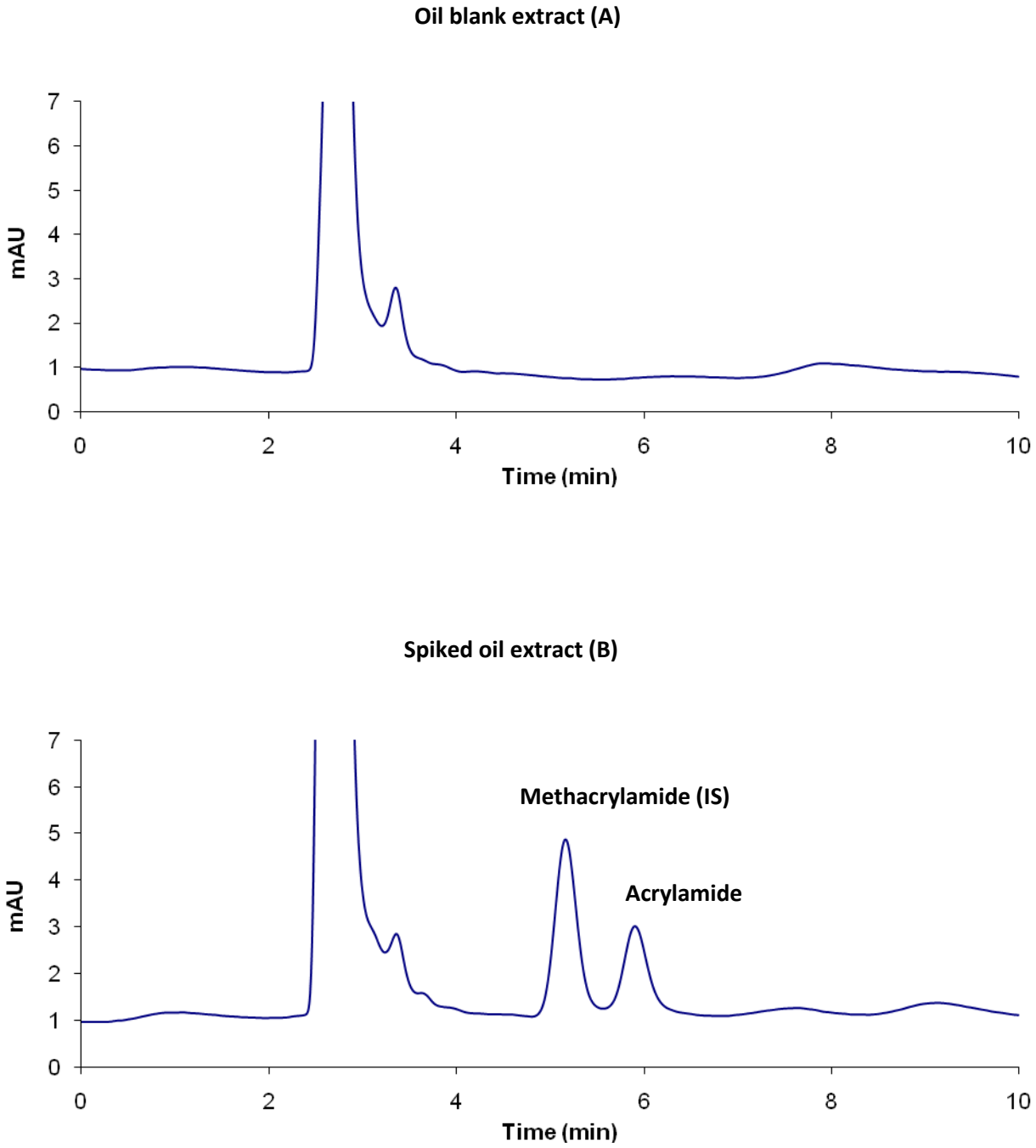



**Figure 5.4.2: Chromatograms of; (A) blank oil extract and (B) spiked oil extract**

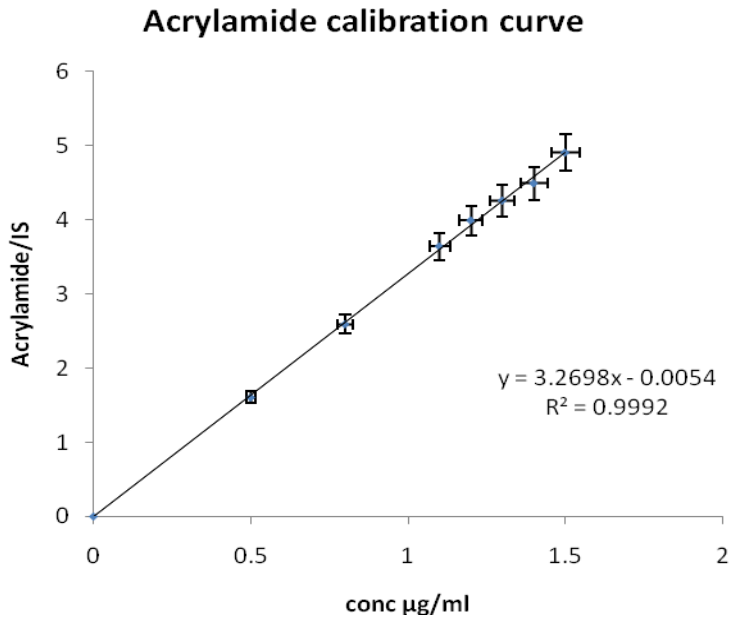



**Figure 5.4.3: Acrylamide calibration curve [LOD = 32.4 ng/ml LOQ = 108 ng/ml**

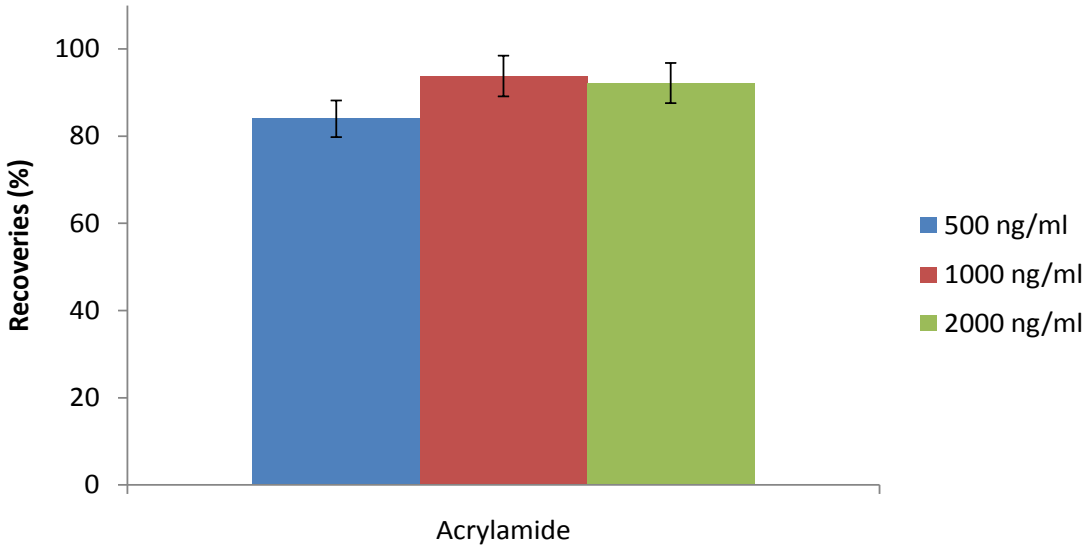



**Figure 5.4.4: Recoveries for the acrylamide in oil sample (n = 6)**

# CONCLUSIONS

This thesis presented an evaluation on the applicability of different types of SPE sorbents. It was demonstrated that the sorbents had the potential to extract analytes of interest from complex matrices. Chromatographic conditions were optimized in order to obtain maximum sensitivity and selectivity. The optimized methods attained excellent results with regards recoveries, reproducibility, linearity, LODs and LOQs.

A simultaneous extraction with subsequent fractionation of acidic, basic and neutral drugs in biological matrices was achieved with relatively simpler SPE protocols. The polymeric SPE sorbents, SampliQ-SAX and SampliQ-SCX allowed for high recoveries (> 80%) with reasonably low RSDs (< 5%, n = 6). The developed methods can therefore be applied for analysis of compounds that exhibit diverse polarity and acidic, basic or neutral functionalities.

Furthermore, the applicability of simple and fast multi-residue methods based on SampliQ QuEChERS was evaluated. QuEChERS method was employed for the determination of sixteen polycyclic aromatic hydrocarbons in soil and fish samples, nine sulfonamides in chicken muscle and acrylamide in cooking oil. High extraction yields (≥ 76%) with excellent RSD (< 5%, n = 6), based on spiked matrices, were attained. These QuEChERS based methodologies maybe applied for quality control concerning PAHs, SAs and AA in real sample and are generally suitable for laboratory routine analysis.

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